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(54) Title: IMPROVEMENT OF T CELL MEDIATED IMMUNITY

(57) Abstract

The present invention provides a method of modifying the T–cell population makeup or increasing the number of T–cells in a subject having depressed or abnormal T–cell population or function, the method comprising disrupting sex steroid signalling to the thymus in the subject. The invention can be used to treat a subject suffering from a wide array of diseases, for example, cancer, HIV infection, autoimmunity and hypersensitivity. In addition, the present invention provides methods for enhancing an immune response to an antigen, treating an autoimmune disease, and decreasing a host–vs–graft reaction in a transplantation donor.

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# IMPROVEMENT OF T CELL MEDIATED IMMUNITY

## FIELD OF THE INVENTION

The present invention concerns methods of modifying the T-cell population make up or increasing the number of T-cells in a subject having depressed or abnormal T-cell population or function. These methods involve disrupting sex steroid signalling to the thymus in the subject.

## BACKGROUND OF THE INVENTION

The thymus is influenced to a great extent by its bidirectional communication with the neuroendocrine system (Kendall, 1988). Of particular importance is the interplay between the pituitary, adrenals and gonads on thymic function including both trophic (TSH and GH) and atrophic effects (LH, FSH and ACTH) (Kendall, 1988; Homo-Delarche, 1991). Indeed one of the characteristic features of thymic physiology is the progressive decline in structure and function which is commensurate with the increase in circulating sex steroid production around puberty (Hirokawa and Makinodan, 1975; Tosi *et al.*, 1982 and Hirokawa, *et al.*, 1994). The precise target of the hormones and the mechanism by which they induce thymus atrophy is yet to be determined. Since the thymus is the primary site for the production and maintenance of the peripheral T cell pool, this atrophy has been widely postulated as the primary cause of an increased incidence of immune-based disorders in the elderly. In particular, deficiencies of the immune system illustrated by a decrease in T-cell dependent immune functions such as cytolytic T-cell activity and mitogenic responses, are reflected by an increased incidence of immunodeficiency, autoimmunity and tumour load in later life (Hirokawa, 1998).

The impact of thymus atrophy is reflected in the periphery, with reduced thymic input to the T cell pool resulting in a less diverse T cell receptor (TCR) repertoire. Altered cytokine profile (Hobbs *et al.*, 1993; Kurashima *et al.*, 1995); changes in $CD4^+$ and $CD8^+$ subsets and a bias towards memory as opposed to naive T cells (Mackall *et al.*, 1995) are also observed. Furthermore, the efficiency of thymopoiesis is impaired with age such that the ability of the immune system to regenerate normal T-cell numbers after T-cell depletion, is eventually lost (Mackall *et al.*, 1995).

However, recent work by Douek *et al.* (1998), has shown presumably thymic output to occur even in old age in humans. Excisional DNA products of TCR gene-rearrangement were used to demonstrate circulating, *de novo* produced naive T cells after HIV infection in older patients. The rate of this output and subsequent peripheral T cell pool regeneration needs to be further addressed since patients who have undergone chemotherapy show a greatly reduced rate of regeneration of the T cell pool, particularly $CD4^+$ T cells, in post-pubertal patients compared to those who were pre-pubertal (Mackall et al, 1995). This is further exemplified in recent work by Timm and Thoman (1999), who have shown that although $CD4^+$ T cells are regenerated in old mice post BMT, they appear to show a bias towards memory cells due to the aged peripheral microenvironment, coupled to poor thymic production of naive T cells.

The thymus essentially consists of developing thymocytes interspersed within the diverse stromal cells (predominantly epithelial cell subsets) which constitute the microenvironment and provide the growth factors and cellular interactions necessary for the optimal development of the T cells. The symbiotic developmental relationship between thymocytes and the epithelial subsets that controls their differentiation and maturation (Boyd *et al.*, 1993), means sex-steroid inhibition could occur at the level of either cell type which would then influence the status of the other. It is less likely that there is an inherent defect within the thymocytes themselves since previous studies, utilising radiation chimeras, have shown that BM stem cells are not affected by age (Hirokawa, 1998; Mackall and Gress, 1997) and have a similar degree of thymus repopulation potential as young BM cells. Furthermore, thymocytes in older aged animals retain their ability to differentiate to at least some degree (Mackall and Gress, 1997; George and Ritter, 1996; Hirokawa *et al.*, 1994). However, recent work by Aspinall (1997), has shown a defect within the precursor $CD3^-CD4^-CD8^-$ triple negative (TN) population occurring at the stage of TCR β chain gene-rearrangement.

The enormous clinical benefits to be gained through restoration of thymic function, would represent an important strategy for the treatment of immunodeficiencies, particularly in the elderly, HIV patients and following chemotherapy.

## SUMMARY OF THE INVENTION

The present inventors have demonstrated that thymic atrophy can be completely reversed by inhibition of sex steroid production, with full restoration of thymic structure and function. The present inventors have also found clinical applications for rejuvenating thymic function by disrupting sex steroid signalling to the thymus.

Accordingly, in a first aspect, the present invention provides a method of modifying the T-cell population makeup or increasing the number of T-cells in a subject having depressed or abnormal T-cell population or function, the method comprising disrupting sex steroid signalling to the thymus in the subject.

Preferably, the modification of T-cell population makeup is characterized by an alteration in the nature and/or ratio of T cell subsets defined functionally and/or by expression of characteristic molecules, wherein the characteristic molecules are selected from the group consisting of: the T cell receptor, CD4, CD8, CD3, CD25, CD28, CD44, CD62L and CD69.

It is further preferred that increasing the number of T-cells in a subject results in a relative increase in T cell numbers when compared to other lymphoid cells. Preferably, the other lymphoid cells are B cells.

It is also preferred that the subject having a depressed or abnormal T-cell population or function is suffering from a condition selected from the group consisting of: cancer, human immunodeficiency virus infection, an autoimmune disease, a hypersensitivity disease or endometriosis.

Preferably, the cancer sufferer has undergone chemotherapy and/or radiation therapy and/or bone marrow transplantation.

Preferably, the subject with the human immunodeficiency virus infection has AIDS.

In a further preferred embodiment, the subject is post-pubertal.

Autoimmune diseases are thought to arise as a polygenic trait, an essential component of which is the participation of pathological self reactive T cells. By treating such subjects with chemotherapy or irradiation, with or without bone marrow transplantation, these self reactive T cells can be ablated. It is envisaged that disruption of sex steroid signalling to the thymus will allow reactivation of the thymus resulting in a cohort of new non-autoreactive T cells.

Accordingly, in a second aspect the present invention provides a method for treating an autoimmune disease in a subject, the method comprising the steps; ablating the resident T cell population, and disrupting sex steroid signalling to the thymus in the subject.

The steps of the second aspect of the present invention can be performed in any order.

In a preferred embodiment, this method further comprises subjecting the individual to a bone marrow transplant.

In a further preferred embodiment, the T cell population is ablated by exposing the individual to chemotherapy or irradiation.

The present invention may also be utilized to enhance an immune response to an antigen in a subject.

Accordingly, in a third aspect the present invention provides a method for enhancing an immune response to an antigen in a subject, the method comprising disrupting sex steroid signalling to the thymus in the subject, and administering an antigen.

The antigen may be, for example, derived from an infectious agent(s) or from a tumour cell.

In a preferred embodiment of the third aspect, the subject is suffering from cancer or an infection.

In a further preferred embodiment of the third aspect, the antigen is mixed with an adjuvant before administration.

In a fourth aspect, the present invention provides a method of decreasing host-vs-graft reaction in a subject following transplantation of an organ, the method comprising the following steps:

ablating T-cells in the subject;

disrupting sex steroid signalling to the thymus in the subject; and

transplanting an organ from a donor into the subject.

Preferably, the method of the fourth aspect also comprises transplanting bone marrow to the subject from the donor.

With respect to each of the methods of the present invention, it is preferred that sex steroid signalling to the thymus is disrupted by inhibiting sex steroid production or by blocking a sex steroid receptor(s) within the thymus.

Preferably, inhibition of sex steroid production is achieved by either castration or administration of a sex steroid analogue(s).

Preferred sex steroid analogues include, eulexin, goserelin, leuprolide, dioxalan derivatives such as triptorelin, meterelin, buserelin, histrelin, nafarelin, lutrelin, leuprorelin, and luteinizing hormone-releasing hormone analogues.

Currently, it is preferred that sex steroid analogue is an analogue of luteinizing hormone-releasing hormone. More preferably, the luteinizing hormone-releasing hormone analogue is deslorelin.

In yet another preferred embodiment, the sex steroid analogue(s) is administered by a sustained peptide-release formulation. Preferred sustained peptide-release formulations are provided in WO 98/08533, the entire contents of which are incorporated herein by reference.

In a fifth aspect, the present invention provides a composition for enhancing an immune response to an antigen in a subject, the composition comprising an adjuvant, the antigen, and an analogue of luteinizing hormone-releasing hormone.

It will also be understood by the skilled addressee that the present invention can be applied to any organism which possesses a thymus at some stage during its development. Preferably, the organism is a mammal. More preferably, the organism is a human.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

## BRIEF DESCRIPTION OF THE ACCOMPANYING FIGURES

**Fig. 1:** Changes in thymocyte number pre- and post-castration. Thymus atrophy results in a significant decrease in thymocyte numbers with age. By 2 weeks post- castration, cell numbers have increased to young adult levels. By 3 weeks post-castration, numbers have significantly increased from the young adult and they are stabilised by 4 weeks post-castration. ***=Significantly different from young adult (2 mth) thymus, $p<0.001$

**Fig. 2:** (A) Spleen numbers remain constant with age and post-castration. The B:T cell ratio in the periphery also remains constant (B),

however, the CD4:CD8 ratio decreases significantly ($p<0.001$) with age and is restored to normal young levels by 4 weeks post-ex.

**Fig. 3**: FACS profiles of CD4 vs. CD8 thymocyte populations with age and post-castration. Percentages for each quadrant are given above each plot. Subpopulations of thymocytes remain constant with age and there is a synchronous expansion of thymocytes following castration.

**Fig. 4.1:** Proliferation of thymocytes as detected by incorporation of a pulse of BrdU. Proportion of proliferating thymocytes remains constant with age and following castration.

**Fig. 4.2**: Effects of age and castration on proliferation of thymocyte subsets. (A) Proportion of each subset that constitutes the total proliferating population. The proportion of CD8+ T cells within the proliferating population is significantly increased. (B) Percentage of each subpopulation that is proliferating. The TN and CD8 Subsets have significantly less proliferation at 2 years than at 2 months. At 2 weeks post-castration, the TN population has returned to normal young levels of proliferation while the CD8 population shows a significant increase in proliferation. The level is equivalent to the normal young by 4 weeks post-castration. (C) Overall TN proliferation remains constant with age and post-castration, however, the significant decrease in proliferation of the TN1 subpopulation with age, is not returned to normal levels by 4 weeks post-castration (D).***=Highly significant, $p<0.001$,**=significant, $p<0.01$

**Fig. 5**: Migration rates from 1 year and 2 year mice as determined by IT FITC labelling. Young adult migration rates are 1% per day. Controls used were non-injected animals. Migration rates remain constant with age.

**Fig. 6**: Changes in thymus, spleen and lymph node cell numbers following treatment with cyclophosphamide, a chemotherapy agent. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate (cyclo alone) group at 1 and 2 weeks post-treatment. In addition, spleen and lymph node numbers of the castrate group were well

increased compared to the cyclophosphamide alone group. By 4 weeks, cell numbers are normalised. (n = 3-4 per treatment group and time point).

**Fig. 7**: Changes in thymus, spleen and lymph node cell numbers following irradiation (625 Rads). Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate (irradiation alone) group at 1 and 2 weeks post-treatment. By 4 weeks, cell numbers are normalised. (n = 3-4 per treatment group and time point).

**Fig. 8**: Changes in thymus, spleen and lymph node cell numbers following irradiation. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate group at 1 and 2 weeks post-treatment. However, the difference observed is not as obvious as when mice were castrated 1 week prior to treatment (Fig. 8). By 4 weeks, cell numbers are normalised. (n = 3-4 per treatment group and time point).

**Fig. 9**: Changes in thymus, spleen and lymph node cell numbers following treatment with cyclophosphamide, a chemotherapy agent. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate (cyclophosphamide alone) group at 1 and 2 weeks post-treatment. In addition, spleen and lymph node numbers of the castrate group were well increased compared to the cyclophosphamide alone group. By 4 weeks, cell numbers are normalised. (n = 3-4 per treatment group and time point). Chemical castration is comparable to surgical castration in regeneration of the immune system post-cyclophosphamide treatment.

**Fig. 10**: Lymph node cellularity following foot-pad immunisation with HSV-1. Note the increased cellularity in the aged post-castration as compared to the non-castrated group. Bottom graph illustrates the overall activated cell number as gated on CD25 vs. CD8 cells by FACS.

**Fig. 11**: Examples of Flow cytometry dot plots illustrating activated cell proportions in lymph nodes following HSV-1 immunisation. Activated cells are CD25/CD8 double-positive.

Fig. 12: Vβ10 expression on CTL in activated LN following HSV-1 inoculation. Note the dimunition of a clonal response in aged mice and the reinstation of the expected response post-castration.

Fig. 13: Changes in thymus, spleen, lymph node and bone marrow cell numbers following bone marrow transplantation of Ly5 congenic mice. Note the rapid expansion of the thymus in castrated animals when compared to the non-castrate group at all time points post-treatment. In addition, spleen and lymph node numbers of the castrate group were well increased compared to the cyclophosphamide alone group. (n = 3-4 per treatment group and time point). Castrated mice had significantly increased congenic (Ly5.2) cells compared to non-castrated animals (data not shown).

Fig. 14: Changes in thymus cell number in castrated and noncastrated mice after foetal liver reconstitution. n = 3-4 for each test group. (A) At two weeks, thymus cell number of castrated mice was at normal levels and significantly higher than that of noncastrated mice ($*p \leq 0.05$). Hypertrophy was observed in thymii of castrated mice after four weeks. Noncastrated cell numbers remain below control levels. (B) $CD45.2^+$ cells-$CD45.2^+$ is a marker showing donor derivation. Two weeks after reconstitution donor-derived cells were present in both castrated and noncastrated mice. Four weeks after treatment approximately 85% of cells in the castrated thymus were donor-derived. There were no donor-derived cells in the noncastrated thymus.

Fig. 15: FACS profiles of CD4 versus CD8 donor derived thymocyte populations after lethal irradiation and foetal liver reconstitution. Percentages for each quadrant are given to the right of each plot. The age matched control profile is of an eight month old Ly5.1 congenic mouse thymus. Those of castrated and noncastrated mice are gated on $CD45.2^+$ cells, showing only donor derived cells. Two weeks after reconstitution subpopulations of thymocytes do not differ between castrated and noncastrated mice.

Fig. 16: Myeloid and lymphoid dendritic cell (DC) number after lethal irradiation, foetal liver reconstitution and castration. n= 3-4 mice for each test group. Control (white) bars on the following graphs are based on the

normal number of dendritic cells found in untreated age matched mice. (A) Donor-derived myeloid dendritic cells-Two weeks after reconstitution DC were present at normal levels in noncastrated mice. There were significantly more DC in castrated mice at the same time point. ($*p \leq 0.05$). At four weeks DC number remained above control levels in castrated mice. (B) Donor-derived lymphoid dendritic cells - Two weeks after reconstitution DC numbers in castrated mice were double those of noncastrated mice. Four weeks after treatment DC numbers remained above control levels.

**Fig. 17**: Changes in total and $CD45.2^+$ bone marrow cell numbers in castrated and noncastrated mice after foetal liver reconstitution. n=3-4 mice for each test group. (A) Total cell number - Two weeks after reconstitution bone marrow cell numbers had normalised and there was no significant difference in cell number between castrated and noncastrated mice. Four weeks after reconstitution there was a significant difference in cell number between castrated and noncastreated mice ($*p \leq 0.05$). (B) $CD45.2^+$ cell number - There was no significant difference between castrated and noncastrated mice with respect to $CD45.2^+$ cell number in the bone marrow, two weeks after reconstitution. $CD45.2^+$ cell number remained high in castrated mice at four weeks. There were no donor-derived cells in the noncastrated mice at the same time point.

**Fig. 18**: Changes in T cells and myeloid and lymphoid derived dendritic cells (DC) in the bone marrow after foetal liver reconstitution. n=3-4 mice for each test group. Control (white bars on the following graphs are based on the normal number of T cells and dendritic cells found in untreated age matched mice. (A) T cell number - Numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived myeloid dendritic cells - Two weeks after reconstitution DC cell numbers were normal in both castrated and noncastrated mice. At this time point there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived lymphoid dendritic cells - Numbers were at normal levels two and four weeks after reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

**Fig. 19**: Change in total and CD45.2$^+$ spleen cell numbers in castrated and noncastrated mice after foetal liver reconstitution. n=3-4 mice for each test group. (A) Total cell number - Two weeks after reconstitution cell numbers were decreased and there was no significant difference in cell number between castrated and noncastrated mice. Four weeks after reconstitution cell numbers were approaching normal levels in castrated mice. (B) CD45.2$^+$ cell number- there was no significant difference between castrated and noncastrated mice with respect to CD45.2$^+$ cell number in the spleen, two weeks after reconstitution. CD45.2$^+$ cell number remained high in castrated mice at four weeks. There were no donor-derived cells in the noncastrated mice at the same time point.

**Fig. 20:** Splenic changes in T cells and myeloid and lymphoid derived dendritic cells (DC) after foetal liver reconstitution. n=3-4 mice for each test group. Control (white) bars on the following graphs are based on the normal number of T cells and dendritic cells found in untreated age matched mice. (A) T cell number - Numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived myeloid dendritic cells - two and four weeks after reconstitution DC numbers were normal in both castrated and noncastrated mice. At two weeks there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived lymphoid dendritic cells - numbers were at normal levels two and four weeks after reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

**Fig. 21**: Changes in total and CD45.2$^+$ lymph node cell numbers in castrated and noncastrated mice after foetal liver reconstitution. n=3-4 for each test group. (A) Total cell numbers - two weeks after reconstitution cell numbers were at normal levels and there was no significant difference in cell number between castrated and noncastrated mice. Four weeks after reconstitution cell numbers in castrated mice were at normal levels. (B) CD45.2$^+$ cell number - There was no significant difference between castrated and noncastrated mice with respect to CD45.2$^+$ cell number in the lymph node, two weeks after reconstitution. CD45.2 cell number remained high in castrated mice at four weeks. There were no donor-derived cells in the noncastrated mice at the same point.

Fig. 22: Changes in T cells and myeloid and lymphoid derived dendritic cells (DC) in the mesenteric lymph nodes after foetal liver reconstitution. n=3-4 mice for each test group. Control (white) bars on the following graphs are based on the normal number of T cells and dendritic cells found in untreated age matched mice. (A) T cell number - Numbers were reduced two and four weeks after reconstitution in both castrated and noncastrated mice. (B) Donor derived myeloid dendritic cells - Two weeks after reconstitution DC numbers were normal in both castrated and noncastrated mice. At four weeks they were decreased. At two weeks there was no significant difference between numbers in castrated and noncastrated mice. (C) Donor-derived lymphoid dendritic cells - numbers were at normal levels two and four weeks after reconstitution. At two weeks there was no significant difference between numbers in castrated and noncastrated mice.

## DETAILED DESCRIPTION OF THE INVENTION

The phrase "modifying the T-cell population makeup" refers to altering the nature and/or ratio of T cell subsets defined functionally and by expression of characteristic molecules. Examples of these characteristic molecules include, but are not limited to, the T cell receptor, CD4, CD8, CD3, CD25, CD28, CD44, CD62L and CD69.

The phrase "increasing the number of T-cells" refers to an absolute increase in the number of T cells in a subject in the thymus and/or in circulation and/or in the spleen and/or in the bone marrow and/or in peripheral tissues such as lymph nodes, gastrointestinal, urogenital and respiratory tracts. This phrase also refers to a relative increase in T cells, for instance when compared to B cells.

A "subject having a depressed or abnormal T-cell population or function" includes an individual suffering from cancer, especially one who has undergone chemotherapy or radiation therapy, or has been subjected to a bone marrow transplant, or breast and prostate cancer patients, or any cancer or proliferative disorder resulting in T cell abnormalities or reduced functional capacity of cell-mediated immunity. This phrase also includes an individual infected with the human immunodeficiency virus, especially one who has AIDS. Furthermore, this phrase includes any post-pubertal

individual, especially an aged person who has decreased immune responsiveness and increased incidence of disease as a consequence of post-pubertal thymic atrophy. This phrase also includes a subject suffering from endometriosis, an autoimmune disease, allergies, hypersensitivities, or any immune dysfunction. The subject may have undergone an allogeneic bone marrow transplantation, or be a post-chemotherapy leukaemia patient such as CLL and low grade Non-Hogkins lymphoma patients treated with drugs such as Fludarabine, cladrabine, dexamethasone and 2-cytodeoxyadenosine which are severely toxic for T cells.

"Adjuvant" means one or more substances that enhances the immunogenicity and efficacy of an antigen composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other oils of animal origin); block copolymers; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; Corynebacterium-derived adjuvants such as corynebacterium parvum; Propionibacterium-derived adjuvants such as Propionibacterium acne; Mycobacterium bovis (Bacillus Calmetic and Guerinn or BCG); interleukins such as interleukin 2 and interleukin-12; monokines such as interleukin 1; tumor necrosis factor; interferons such as gamma interferon; combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; liposomes; iscom adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as murarnyl dipeptides or other derivatives; Avridine; Lipid A; dextran sulfate; DEAE-Dextran or DHAE-Dextran with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (e.g. U.S. Pat. No. 5,047,238); vaccinia or animal virus proteins; sub-viral particle adjuvants such as cholera toxin, or mixtures thereof.

With regard to the method of decreasing host-vs-graft reaction in a subject following transplantation of an organ, the host lymphocytes would be first depleted (eg through irradiation or chemotherapy). This could be followed by donor bone marrow/stem cell transplantation linked with disruption of sex steroid signalling to the thymus, to establish chimeras which would include establishment of donor cells including dendritic cells in the host thymus to cause tolerance of newly developed host T cells to the donor. After establishment of the central tolerance, the host would receive a graft from the donor of the stem cells.

As will be readily understood, sex steroid signalling to the thymus can be disrupted in a range of ways, for example, inhibition of sex steroid production or blocking a sex steroid receptor(s) within the thymus. Inhibition of sex steroid production can be achieved, for example, by castration, administration of a sex steroid analogue(s), and other well known techniques. In some clinical cases permanent removal of the gonads via physical castration may be appropriate. In a preferred embodiment, the sex steroid signalling to the thymus is disrupted by administration of a sex steroid analogue, preferably an analogue of luteinizing hormone-releasing hormone. It is currently preferred that the analogue is deslorelin (described in U.S. Patent No. 4218439).

Sex steroid analogues and their use in therapies and "chemical castration" are well known. Examples of such analogues include Eulexin (described in FR7923545, WO 86/01105 and PT100899), Goserelin (described in US4100274, US4128638, GB9112859 and GB9112825), Leuprolide (described in US4490291, US3972859, US4008209, US4005063, DE2509783 and US4992421), dioxalan derivatives such as are described in EP 413209, Triptorelin (described in US4010125, US4018726, US4024121, EP 364819 and US5258492), Meterelin (described in EP 23904), Buserelin (described in US4003884, US4118483 and US4275001), Histrelin (described in EP217659), Nafarelin (described in US4234571, WO93/15722 and EP52510), Lutrelin (described in US4089946), Leuprorelin (described in Plosker *et al.*) and LHRH analogues such as are described in EP181236, US4608251, US4656247, US4642332, US4010149, US3992365 and US4010149. The disclosures of each the references referred to above are incorporated herein by cross reference.

As will be understood by persons skilled in the art at least some of the means for disrupting sex steroid signalling to the thymus will only be effective as long as the appropriate compound is administered. As a result, an advantage of certain embodiments of the present invention is that once the desired immunological affects of the present invention have been achieved, (2-3 months) the treatment can be stopped and the subjects reproductive system will return to normal.

As will be understood, the term "organ" is used in its broadest sense and includes skin, kidney, liver, heart, lung etc.

## EXAMPLES

## EXAMPLE 1 - REVERSAL OF AGED-INDUCED THYMIC ATROPHY

### Materials and Methods

**Animals**

CBA/CAH and C57Bl6/J male mice were obtained from Central Animal Services, Monash University and were housed under conventional conditions. Ages ranged from 4-6 weeks to 26 months of age and are indicated where relevant.

**Castration**

Animals were anaesthetised by intraperitoneal injection of 0.3ml of 0.3mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and 1.5mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline. Surgical castration was performed by a scrotal incision, revealing the testes, which were tied with suture and then removed along with surrounding fatty tissue.

**Bromodeoxyuridine (BrdU) incorporation**

Mice received two intraperitoneal injections of BrdU (Sigma Chemical Co., St. Louis, MO) (100mg/kg body weight in 100µl of PBS) at a 4 hour interval. Control mice received vehicle alone injections. One hour after the second injection, thymuses were dissected and either a cell suspension made for FACS analysis, or immediately embedded in Tissue Tek (O.C.T. compound, Miles INC, Indiana), snap frozen in liquid nitrogen, and stored at –70°C until use.

**Flow Cytometric analysis**

Mice were killed by $CO_2$ asphyxiation and thymus, spleen and mesenteric lymph nodes were removed. Organs were pushed gently through a 200µm sieve in cold PBS/1% FCS/0.02% Azide, centrifuged (650g, 5 min, 4°C), and resuspended in either PBS/FCS/Az. Spleen cells were incubated in red cell lysis buffer (8.9g/litre ammonium chloride) for 10 min at 4°C, washed and resuspended in PBS/FCS/Az. Cell concentration and viability were determined in duplicate using a haemocytometer and ethidium

bromide/acridine orange and viewed under a fluorescence microscope (Axioskop; Carl Zeiss, Oberkochen, Germany).

For 3-colour immunofluorescence thymocytes were routinely labelled with anti-αβ TCR-FITC or anti-γδ TCR-FITC, anti-CD4-PE and anti-CD8-APC (all obtained from Pharmingen, San Diego, CA) followed by flow cytometry analysis. Spleen and lymph node suspensions were labelled with either αβTCR-FITC/CD4-PE/CD8-APC or B220-B (Sigma) with CD4-PE and CD8-APC. B220-B was revealed with streptavidin-Tri-color conjugate purchased from Caltag Laboratories, Inc., Burlingame, CA.

For BrdU detection, cells were surface labelled with CD4-PE and CD8-APC, followed by fixation and permeabilisation as previously described (Carayon and Bord, 1989). Briefly, stained cells were fixed O/N at 4°C in 1% PFA/0.01% Tween-20. Washed cells were incubated in 500µl DNase (100 Kunitz units, Boehringer Mannheim, W. Germany) for 30 mins at 37°C in order to denature the DNA. Finally, cells were incubated with anti-BrdU-FITC (Becton-Dickinson).

For 4-colour Immunofluorescence thymocytes were labelled for CD3, CD4, CD8, B220 and Mac-1, collectively detected by anti-rat Ig-Cy5 (Amersham, U.K.), and the negative cells (TN) gated for analysis. They were further stained for CD25-PE (Pharmingen) and CD44-B (Pharmingen) followed by Streptavidin-Tri-colour (Caltag, CA) as previously described (Godfrey and Zlotnik, 1993). BrdU detection was then performed as described above.

Samples were analysed on a FacsCalibur (Becton-Dickinson). Viable lymphocytes were gated according to 0° and 90° light scatter profiles and data was analysed using Cell quest software (Becton-Dickinson).

**Immunohistology**

Frozen thymus sections (4µm) were cut using a cryostat (Leica) and immediately fixed in 100% acetone.

For two-colour immunofluorescence, sections were double-labelled with a panel of monoclonal antibodies: MTS6, 10, 12, 15, 16, 20, 24, 32, 33, 35 and 44 (Godfrey *et al.*, 1990; Table 1) produced in this laboratory and the co-expression of epithelial cell determinants was assessed with a polyvalent rabbit anti-cytokeratin Ab (Dako, Carpinteria, CA). Bound mAb was revealed with FITC-conjugated sheep anti-rat Ig (Silenus Laboratories) and anti-

cytokeratin was revealed with TRITC-conjugated goat anti-rabbit Ig (Silenus Laboratories).

For bromodeoxyuridine detection sections were stained with either anti-cytokeratin followed by anti-rabbit-TRITC or a specific mAb, which was then revealed with anti-rat Ig-Cy3 (Amersham). BrdU detection was then performed as previously described (Penit *et al.*, 1996). Briefly, sections were fixed in 70% Ethanol for 30 mins. Semi-dried sections were incubated in 4M HCl, neutralised by washing in Borate Buffer (Sigma), followed by two washes in PBS. BrdU was detected using anti-BrdU-FITC (Becton-Dickinson).

For three-colour immunofluorescence, sections were labelled for a specific MTS mAb together with anti-cytokeratin. BrdU detection was then performed as described above.

Sections were analysed using a Leica fluorescent and Nikon confocal microscopes.

**Migration studies**

Animals were anaesthetised by intraperitoneal injection of 0.3ml of 0.3mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and 1.5mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline.

Details of the FITC labelling of thymocytes technique are similar to those described elsewhere (Scollay *et al.*, 1980; Berzins *et al.*, 1998). Briefly, thymic lobes were exposed and each lobe was injected with approximately 10μm of 350 μg/ml FITC (in PBS). The wound was closed with a surgical staple, and the mouse was warmed until fully recovered from anaesthesia. Mice were killed by $CO_2$ asphyxiation approximately 24h after injection and lymphoid organs were removed for analysis.

After cell counts, samples were stained with anti-CD4-PE and anti-CD8-APC, then analysed by flow cytometry. Migrant cells were identified as live-gated $FITC^+$ cells expressing either CD4 or CD8 (to omit autofluorescing cells and doublets). The percentages of $FITC^+$ CD4 and CD8 cells were added to provide the total migrant percentage for lymph nodes and spleen, respectively. Calculation of daily export rates was performed as described by Berzins *et al.* (1998).

Data was analysed using the unpaired student 't' test or nonparametrical Mann-Whitney test was used to determine the statistical significance between control and test results for experiments performed at least in triplicate. Experimental values significantly differing from control values are indicated as follows: *p≤0.05, **p≤0.01 and ***p≤0.001.

## Results

**The effect of age on thymocyte populations.**

(i) *Thymic weight and thymocyte number*

With increasing age there is a highly significant (p≤0.0001) decrease in both thymic weight (Figure 1A) and total thymocyte number (Figure 1B). Relative thymic weight (mg thymus/g body) in the young adult has a mean value of 3.34 which decreases to 0.66 at 18-24 months of age (adipose deposition limits accurate calculation). The decrease in thymic weight can be attributed to a decrease in total thymocyte numbers: the 1-2 month thymus contains ~$6.7 \times 10^7$ thymocytes, decreasing to ~$4.5 \times 10^6$ cells by 24 months. By removing the effects of sex steroids on the thymus by castration, regeneration occurs and by 4 weeks post-castration, the thymus is equivalent to that of the young adult in both weight and cellularity (Figure 1A and 1B). Interestingly, there is a significant (p≤0.001) increase in thymocyte numbers at 2 weeks post-castration (~$1.2 \times 10^8$), which is restored to normal young levels by 4 weeks post-castration (Figure 1B).

The decrease in T cell numbers produced by the thymus is not reflected in the periphery, with spleen cell numbers remaining constant with age (Figure 2A). Homeostatic mechanisms in the periphery were evident since the B cell to T cell ratio in spleen and lymph nodes was not affected with age and the subsequent decrease in T cell numbers reaching the periphery (Figure 2B). However, the ratio of $CD4^+$ to $CD8^+$ T cell significantly decreased (p≤0.001) with age from 2:1 at 2 months of age, to a ratio of 1:1 at 2 years of age (Figure 2C). Following castration and the subsequent rise in T cell numbers reaching the periphery, no change in peripheral T cell numbers was observed: splenic T cell numbers and the ratio of B:T cells in both spleen and lymph nodes was not altered following castration (Figure 2A and B). The decreased CD4:CD8 ratio in the periphery with age was still evident at 2 weeks post-castration but was completely reversed by 4 weeks post-castration (Figure 2C).

(ii) *αβTCR, γδTCR, CD4 and CD8 expression*

To determine if the decrease in thymocyte numbers seen with age was the result of the depletion of specific cell populations, thymocytes were labelled with defining markers in order to analyse the separate subpopulations. In addition, this allowed analysis of the kinetics of thymus repopulation post-castration. The proportion of the main thymocyte subpopulations was compared with those of the normal young thymus (Figure 3) and found to remain uniform with age. In addition, further subdivision of thymocytes by the expression of αβTCR and γδTCR revealed no change in the proportions of these populations with age (data not shown). At 2 and 4 weeks post-castration, thymocyte subpopulations remained in the same proportions and, since thymocyte numbers increase by up to 100-fold post-castration, this indicates a synchronous expansion of all thymocyte subsets rather than a developmental progression of expansion.

The decrease in cell numbers seen in the thymus of aged animals thus appears to be the result of a balanced reduction in all cell phenotypes, with no significant changes in T cell populations being detected. Thymus regeneration occurs in a synchronous fashion, replenishing all T cell subpopulations simultaneously rather than sequentially.

**Proliferation of thymocytes**

As shown in Figure 4.1, 15-20% of thymocytes are proliferating at 4-6 weeks of age. The majority (~80%) of these are DP with the TN subset making up the second largest population at ~6% (Figure 4.2A). Accordingly, most division is seen in the subcapsule and cortex by immunohistology (data not shown). Some division is seen in the medullary regions with FACS analysis revealing a proportion of SP cells (9% of CD4 T cells and 25% of CD8 T cells) dividing (Figure 4.2B).

Although cell numbers are significantly decreased in the aged thymus, proliferation of thymocytes remains constant, decreasing to 12-15% at 2 years (Figure 4.1), with the phenotype of the proliferating population resembling the 2 month thymus (Figure 4.2A). Immunohistology revealed the division at 1 year of age to reflect that seen in the young adult, however, at 2 years, proliferation is mainly seen in the outer cortex and surrounding the vasculature (data not shown). At 2 weeks post-castration, although

thymocyte numbers significantly increase, there is no change in the proportion of thymocytes that are proliferating, again indicating a synchronous expansion of cells (Figure 4.1). Immunohistology revealed the localisation of thymocyte proliferation and the extent of dividing cells to resemble the situation in the 2-month-old thymus by 2 weeks post-castration (data not shown). When analysing the proportion of each subpopulation which represent the proliferating population, there was a significant ($p<0.001$) increase in the percentage of CD8 T cells which are within the proliferating population (1% at 2 months and 2 years of age, increasing to ~6% at 2 weeks post-castration) (Figure 4.2A).

Figure 4.2B illustrates the extent of proliferation within each subset in young, old and castrated mice. There is a significant ($p\leq0.001$) decay in proliferation within the DN subset (35% at 2 months to 4% by 2 years). Proliferation of $CD8^+$ T cells was also significantly ($p\leq0.001$) decreased, reflecting the findings by immunohistology (data not shown) where no division is evident in the medulla of the aged thymus. The decrease in DN proliferation is not returned to normal young levels by 4 weeks post-castration. However, proliferation within the $CD8^+$ T cell subset is significantly ($p\leq0.001$) increased at 2 weeks post-castration and is returning to normal young levels at 4 weeks post-castration.

The decrease in proliferation within the DN subset was analysed further using the markers CD44 and CD25. The DN subpopulation, in addition to the thymocyte precursors, contains $\alpha\beta TCR^+CD4^-CD8^-$ thymocytes, which are thought to have downregulated both co-receptors at the transition to SP cells (Godfrey & Zlotnik, 1993). By gating on these mature cells, it was possible to analyse the true TN compartment ($CD3^-CD4^-CD8^-$) and these showed no difference in their proliferation rates with age or following castration (Figure 4.2C). However, analysis of the subpopulations expressing CD44 and CD25, showed a significant ($p<0.001$) decrease in proliferation of the TN1 subset ($CD44^+CD25^-$), from 20% in the normal young to around 6% at 18 months of age (Figure 4.2D) which was restored by 4 weeks post-castration. The decrease in the proliferation of the TN1 subset, was compensated for by a significant ($p\leq0.001$) increase in proliferation of the TN2 subpopulation ($CD44^+CD25^+$) which returned to normal young levels by 2 weeks post-castration (Figure 4.2D).

**The effect of age on the thymic microenvironment.**

The changes in the thymic microenvironment with age were examined by immunofluorescence using an extensive panel of mAbs from the MTS series, double-labelled with a polyclonal anti-cytokeratin Ab.

The antigens recognised by these mAbs can be subdivided into three groups: thymic epithelial subsets, vascular-associated antigens and those present on both stromal cells and thymocytes.

(i) *Epithelial cell antigens.*

Anti-keratin staining (pan-epithelium) of 2 year old mouse thymus, revealed a loss of general thymus architecture with a severe epithelial cell disorganisation and absence of a distinct cortico-medullary junction. Further analysis using the mAbs, MTS 10 (medulla) and MTS44 (cortex), showed a distinct reduction in cortex size with age, with a less substantial decrease in medullary epithelium (data not shown). Epithelial cell free regions, or keratin negative areas (KNA's, van Ewijk *et al.*, 1980; Godfrey *et al.*, 1990; Bruijntjes *et al.*, 1993).) were more apparent and increased in size in the aged thymus, as evident with anti-cytokeratin labelling. There is also the appearance of thymic epithelial "cyst-like" structures in the aged thymus particularly noticeable in medullary regions (data not shown). Adipose deposition, severe decrease in thymic size and the decline in integrity of the cortico-medullary junction are shown conclusively with the anti-cytokeratin staining (data not shown). As shown in Figure 2.1, the thymus is beginning to regenerate by 2 weeks post-castration. This is evident in the size of the thymic lobes (a), the increase in cortical epithelium as revealed by MTS 44 (b) and the localisation of medullary epithelium (c). The medullary epithelium is detected by MTS 10 and at 2 weeks, there are still subpockets of epithelium stained by MTS 10 scattered throughout the cortex. By 4 weeks post-castration, there is a distinct medulla and cortex and discernible cortico-medullary junction (data not shown).

The markers MTS 20 and 24 are presumed to detect primordial epithelial cells (Godfrey, *et al.*, 1990) and further illustrate the degeneration of the aged thymus. These are present in abundance at E14, detect isolated medullary epithelial cell clusters at 4- 6 weeks but are again increased in intensity in the aged thymus (data not shown). Following castration, all these antigens are expressed at a level equivalent to that of the young adult

thymus (data not shown) with MTS 20 and MTS 24 reverting to discrete subpockets of epithelium located at the cortico-medullary junction.

(ii) *Vascular-associated antigens.*

The blood-thymus barrier is thought to be responsible for the immigration of T cell precursors to the thymus and the emigration of mature T cells from the thymus to the periphery.

The mAb MTS 15 is specific for the endothelium of thymic blood vessels, demonstrating a granular, diffuse staining pattern (Godfrey, *et al,* 1990). In the aged thymus, MTS 15 expression is greatly increased, and reflects the increased frequency and size of blood vessels and perivascular spaces (data not shown).

The thymic extracellular matrix, containing important structural and cellular adhesion molecules such as collagen, laminin and fibrinogen, is detected by the mAb MTS 16. Scattered throughout the normal young thymus, the nature of MTS 16 expression becomes more widespread and interconnected in the aged thymus. Expression of MTS 16 is increased further at 2 weeks post-castration while 4 weeks post-castration, this expression is representative of the situation in the 2 month thymus (data not shown).

(iii) *Shared antigens*

MHC II expression in the normal young thymus, detected by the mAb MTS 6, is strongly positive (granular) on the cortical epithelium (Godfrey *et al.*, 1990) with weaker staining of the medullary epithelium. The aged thymus shows a decrease in MHCII expression with expression substantially increased at 2 weeks post-castration. By 4 weeks post-castration, expression is again reduced and appears similar to the 2 month old thymus (data not shown).

**Thymocyte emigration**

Approximately 1% of T cells migrate from the thymus daily in the young mouse (Scollay *et al.*, 1980). We found migration was occurring at a proportional rate equivalent to the normal young mouse at 14 months and even 2 years of age (Figure 5) although significantly ($p \leq 0.0001$) reduced in number. There was an increase in the CD4:CD8 ratio of the recent thymic emigrants from ~3:1 at 2 months to ~7:1 at 26 months. By 1 week post-

castration, cell number migrating to the periphery has substantially increased with the overall rate of migration remaining constant at 1-1.5%.

**EXAMPLE 2 - REVERSAL OF CHEMOTHERAPY- OR RADIATION-INDUCED THYMIC ATROPHY.**

Castrated mice (either one-week prior to treatment, or on the same day as treatment), showed substantial increases in thymus regeneration rate following irradiation or cyclophosphamide treatment.

In the thymus, irradiated mice show severe disruption of thymic architecture, concurrent with depletion of rapidly dividing cells. Cortical collapse, reminiscent of the aged/hydrocortisone treated thymus, reveals loss of DN and DP thymocytes. There is a downregulation of αβ-TCR expression on CD4+ and CD8+ SP thymocytes - evidence of apoptosing cells. In comparison, cyclophosphamide-treated animals show a less severe disruption of thymic architecture, and show a faster regeneration rate of DN and DP thymocytes.

By 1 week post-treatment castrated mice showed significant thymic regeneration even at this early stage (Figures 6, 7 and 8). In comparison, non-castrated animals, showed severe loss of DN and DP thymocytes (rapidly-dividing cells) and subsequent increase in proportion of CD4 and CD8 cells (radio-resistant). This is best illustrated by the differences in thymocyte numbers with castrated animals showing at least a 4-fold increase in thymus size even at 1 week post-treatment. By 2 weeks, the non-castrated animals showed relative thymocyte normality with regeneration of both DN and DP thymocytes. However, proportions of thymocytes are not yet equivalent to the young adult control thymus. Indeed, at 2 weeks, the vast difference in regulation rates between castrated and non-castrated mice was maximal (by 4 weeks thymocyte numbers were equivalent between treatment groups).

Interestingly, thymus size appears to 'overshoot' the baseline of the control thymus. Indicative of rapid expansion within the thymus, with the migration of these newly derived thymocytes not yet occurring (it takes ~3-4 weeks for thymocytes to migrate through and out into the periphery). Therefore, although proportions within each subpopulation are equal, numbers of thymocytes are building before being released into the periphery.

Figure 9 illustrates the use of chemical castration compared to surgical castration in enhancement of T cell regeneration. The kinetics of chemical castration are much slower than surgical, that is, mice take about 3 weeks longer to decrease their circulating sex steroid levels. However, chemical castration is still effective in regenerating the thymus as illustrated in Figure 9.

**EXAMPLE 3 - THYMIC REGENERATION FOLLOWING INHIBITION OF SEX STEROIDS RESULTS IN RESTORATION OF DEFICIENT PERIPHERAL T CELL FUNCTION.**

To determine whether castration can enhance the immune response, Herpes Simplex Virus (HSV) immunisation was examined as it allows the study of disease progression and role of CTL (cytotoxic) T cells. Castrated mice have a qualitatively and quantitatively improved responsiveness to the virus. Mice were immunised in the footpad and the popliteal (draining) lymph node analysed at D5 post-immunisation. In addition, the footpad is removed and homogenised to determine the virus titre at particular time-points throughout the experiment.

At D5 post-immunisation, the castrated mice have a significantly larger lymph node cellularity than the aged mice (Figure 10). In addition, activated cell numbers within the lymph nodes are significantly increased when compared to the aged controls (Figure 10 and 11). Further, activated cell numbers correlate with that found for the young adult indicating that CTLs are being activated to a greater extent in the castrated mice, but the young adult may have an enlarged lymph node due to B cell activation.

There is a 40% bias post-immunisation for Vβ10 usage for the CTLs in response to HSV. When aged and castrated mice were analysed for their Vβ expression, it was found that this was predominant in the young adult and castrated mice. However, no such bias was observed with the aged mice (Figure 12). This illustrates the vital need for increased production of T cells from the thymus throughout life, in order to get maximal immune responsiveness.

## EXAMPLE 4 - INHIBITION OF SEX STEROIDS ENHANCES UPTAKE OF NEW HAEMOPOIETIC PRECURSOR CELLS INTO THE THYMUS WHICH ENABLES CHIMERIC MIXTURES OF HOST AND DONOR LYMPHOID CELLS (T, B, AND DENDRITIC CELLS)

Previous experiments have shown that microchimera formation plays an important role in organ transplant acceptance. Dendritic cells have also been shown to play an integral role in tolerance to graft antigens. Therefore, the effects of castration on thymic chimera formation and dendritic cell number was studied.

For the syngeneic experiments, 4 three month old mice were used per treatment group. All controls were age matched and untreated. For congenic experiments, 3-4 eight month old mice were used per treatment group. All controls were age matched and untreated.

**Thymic changes following lethal irradiation, foetal liver reconstitution and castration of syngeneic mice**

The total thymus cell numbers of castrated and noncastrated reconstituted mice were compared to untreated age matched controls and are summarised in Figure 12. One week after treatment total leukocyte numbers of both castrated and noncastrated mice were lower than the control but did not differ significantly from each other. At 3 weeks cell number remained below control levels, however, those of castrated mice was three fold higher than the noncastrated mice ($p<0.05$) (Figure 13A).

**Splenic changes following lethal irradiation, syngeneic foetal liver reconstitution and castration.**

Total cell numbers in the spleen were greatly decreased 1 and 3 weeks after irradiation and reconstitution, in both castrated and noncastrated mice. There was no statistically significant difference in total spleen cell number between castrated and noncastrated treatment groups (Figure 13B).

**Mesenteric lymph nodes following lethal irradiation, syngeneic foetal liver reconstitution and castration**

Mesenteric lymph node cell numbers were greatly decreased 1 week after irradiation and reconstitution, in both castrated and noncastrated mice. However, by the 3 week time point cell numbers had reached control levels.

There was no statistically significant difference in lymph node cell number between castrated and noncastrated treatment groups (Figure 13C).

**Thymic changes following lethal irradiation, foetal liver reconstitution and castration of congenic mice**

In noncastrated mice, there was a profound decrease in thymocyte number over the 4 week time period, with little or no evidence of regeneration (Figure 14A). In the castrated group, however, by two weeks there was already extensive thymopoiesis which by four weeks had returned to control levels, being 10 fold higher than in noncastrated mice. Flow cytometeric analysis of the thymii with respect to CD45.2 (donor-derived antigen) demonstrated that no donor derived cells were detectable in the noncastrated group at 4 weeks, but remarkably, virtually all the thymocytes in the castrated mice were donor - derived at this time point (Figure 14B). Given this extensive enhancement of thymopoiesis from donor-derived haemopoietic precursors, it was important to determine whether T cell differentiation had proceeded normally. CD4, CD8 and TCR defined subsets were analysed by flow cytometry. There were no proportional differences in thymocytes subset proportions 2 weeks after reconstitution (Figure 15). This observation was not possible at 4 weeks, because the noncastrated mice were not reconstituted with donor derived cells. However, at this time point the thymocyte proportions in castrated mice appear normal.

Two weeks after foetal liver reconstitution there were significantly more donor-derived, myeloid dendritic cells (defined as CD45.2+ Mac1+ CD11C+) in castrated mice than noncastrated mice, the difference was 4-fold ($p<0.05$). Four weeks after treatment the number of donor-derived myeloid dendritic cells remained above the control in castrated mice (Figure 16A). 2 weeks after foetal liver reconstitution the number of donor derived lymphoid dendritic cells (defined as CD45.2+Mac1-CD11C+) in the thymus of castrated mice was double that found in noncastrated mice. Four weeks after treatment the number of donor-derived lymphoid dendritic cells remained above the control in castrated mice (Figure 16B).

Immunofluorescent staining for CD11C, epithelium (antikeratin) and CD45.2 (donor-derived marker) localised dendritic cells to the corticomedullary junction and medullary areas of thymii 4 weeks after reconstitution and castration. Using colocalisation software donor-derivation

of these cells was confirmed (data not shown). This was supported by flow cytometry data suggesting that 4 weeks after reconstitution approximately 85% of cells in the thymus are donor derived.

**Changes in the bone marrow following lethal irradiation, foetal liver reconstitution and castration**

Cell numbers in the bone marrow of castrated and noncastrated reconstituted mice were compared to those of untreated age matched controls and are summarised in Figure 17A. Bone marrow cell numbers were normal two and four weeks after reconstitution in castrated mice. Those of noncastrated mice were normal at two weeks but dramatically decreased at four weeks ($p<0.05$). Although, at this time point the noncastrated mice did not reconstitute with donor-derived cells.

Flow cytometeric analysis of the bone marrow with respect to CD45.2 (donor-derived antigen) established that no donor derived cells were detectable in the bone marrow of noncastrated mice 4 weeks after reconstitution, however, almost all the cells in the castrated mice were donor- derived at this time point (Figure 17B).

Two weeks after reconstitution the donor-derived T cell numbers of both castrated and noncastrated mice were markedly lower than those seen in the control mice ($p<0.05$). At 4 weeks there were no donor-derived T cells in the bone marrow of noncastrated mice and T cell number remained below control levels in castrated mice (Figure 18A).

Donor-derived, myeloid and lymphoid dendritic cells were found at control levels in the bone marrow of noncastrated and castrated mice 2 weeks after reconstitution. Four weeks after treatment numbers decreased further in castrated mice and no donor-derived cells were seen in the noncastrated group (Figure 18B).

**Splenic changes following lethal irradiation, foetal liver reconstitution and castration**

Spleen cell numbers of castrated and noncastrated reconstituted mice were compared to untreated age matched controls and the results are summarised in Figure 19A. Two weeks after treatment, spleen cell numbers of both castrated and noncastrated mice were approximately 50% that of the control. By four weeks, numbers in castrated mice were approaching normal

levels, however, those of noncastrated mice remained decreased. Analysis of CD45.2 (donor-derived) flow cytometry data demonstrated that there was no significant difference in the number of donor derived cells of castrated and noncastrated mice, 2 weeks after reconstitution (Figure 19B). No donor derived cells were detectable in the spleens of noncastrated mice at 4 weeks, however, almost all the spleen cells in the castrated mice were donor derived.

Two and four weeks after reconstitution there was a marked decrease in T cell number in both castrated and noncastrated mice ($p<0.05$) (Figure 20A). Two weeks after foetal liver reconstitution donor-derived myeloid and lymphoid dendritic cells (Figures 20 A and B respectively) were found at control levels in noncastrated and castrated mice. At 4 weeks no donor derived dendritic cells were detectable in the spleens of noncastrated mice and numbers remained decreased in castrated mice.

**The effects of lethal irradiation, foetal liver reconstitution and castration on mesenteric lymph node numbers.**

Lymph node cell numbers of castrated and noncastrated, reconstituted mice were compared to those of untreated age matched controls and are summarised in Figure 21A. Two weeks after reconstitution cell numbers were at control levels in both castrated and noncastrated mice. Four weeks after reconstitution, cell numbers in castrated mice remained at control levels but those of noncastrated mice decreased significantly (Figure 21B). Flow cytometry analysis with respect to CD45.2 suggested that there was no significant difference in the number of donor-derived cells, in castrated and noncastrated mice, 2 weeks after reconstitution (Figure 21B). No donor derived cells were detectable in noncastrated mice 4 weeks after reconstitution. However, virtually all lymph node cells in the castrated mice were donor-derived at the same time point.

Two and four weeks after reconstitution donor-derived T cell numbers in both castrated and noncastrated mice were lower than control levels. At 4 weeks the numbers remained low in castrated mice and there were no donor-derived T cells in the lymph nodes of noncastrated mice (Figure 22). Two weeks after foetal liver reconstitution donor-derived, myeloid and lymphoid dendritic cells were found at control levels in noncastrated and castrated mice (Figures 22 A & B respectively). Four weeks after treatment

the number of donor-derived myeloid dendritic cells fell below the control, however, lymphoid dendritic cell number remained unchanged.

**General Discussion of the Examples**

We have shown that aged thymus, although severely atrophic, maintains its functional capacity with age, with T cell proliferation, differentiation and migration occurring at levels equivalent to the young adult mouse. Although thymic function is regulated by several complex interactions between the neuro-endocrine-immune axes, the atrophy induced by sex steroid production exerts the most significant and prolonged effects illustrated by the extent of thymus regeneration post-castration both of lymphoid and epithelial cell subsets.

Thymus weight is significantly reduced with age as shown previously (Hirokawa and Makinodan, 1975, Aspinall, 1997) and correlates with a significant decrease in thymocyte numbers. The stress induced by the castration technique, which may result in further thymus atrophy due to the actions of corticosteroids, is overridden by the removal of sex steroid influences with the 2-week castrate thymus increasing in cellularity by 20-30 fold from the pre-castrate thymus. By 3 weeks post-castration, the aged thymus shows a significant increase in both thymic size and cell number, surpassing that of the young adult thymus presumably due to the actions of sex steroids already exerting themselves in the 2 month old mouse.

Our data confirms previous findings that emphasise the continued ability of thymocytes to differentiate and maintain constant subset proportions with age (Aspinall, 1997). In addition, we have shown thymocyte differentiation to occur simultaneously post-castration indicative of a synchronous expansion in thymocyte subsets. Since thymocyte numbers are decreased significantly with age, proliferation of thymocytes was analysed to determine if this was a contributing factor in thymus atrophy.

Proliferation of thymocytes was not affected by age-induced thymic atrophy or by removal of sex-steroid influences post-castration with ~14% of all thymocytes proliferating. However, the localisation of this division differed with age: the 2 month mouse thymus shows abundant division throughout the subcapsular and cortical areas (TN and DP T cells) with some division also occurring in the medulla. Due to thymic epithelial

disorganisation with age, localisation of proliferation was difficult to distinguish but appeared to be less uniform in pattern than the young and relegated to the outer cortex. By 2 weeks post-castration, dividing thymocytes were detected throughout the cortex and were evident in the medulla with similar distribution to the 2 month thymus.

The phenotype of the proliferating population as determined by CD4 and CD8 analysis, was not altered with age or following castration. However, analysis of proliferation within thymocyte subpopulations, revealed a significant decrease in proliferation of both the TN and $CD8^+$ cells with age. Further analysis within the TN subset on the basis of the markers CD44 and CD25, revealed a significant decrease in proliferation of the TN1 ($CD44^+CD25^-$) population which was compensated for by an increase in the TN2 ($CD44^-CD25^+$) population. These abnormalities within the TN population, reflect the findings by Aspinall (1997). Surprisingly, the TN subset was proliferating at normal levels by 2 weeks post-castration indicative of the immediate response of this population to the inhibition of sex-steroid action. Additionally, at both 2 weeks and 4 weeks post-castration, the proportion of $CD8^+$ T cells that were proliferating was markedly increased from the control thymus, possibly indicating a role in the re-establishment of the peripheral T cell pool.

Thymocyte migration was shown to occur at a constant proportion of thymocytes with age conflicting with previous data by Scollay et al (1980) who showed a ten-fold reduction in the rate of thymocyte migration to the periphery. The difference in these results may be due to the difficulties in intrathymic FITC labelling of 2 year old thymuses or the effects of adipose deposition on FITC uptake. However, the absolute numbers of T cells migrating was decreased significantly as found by Scollay resulting in a significant reduction in ratio of RTEs to the peripheral T cell pool. This will result in changes in the periphery predominantly affecting the T cell repertoire (Mackall *et al.*, 1995). Previous papers (Mackall et al, 1995) have shown a skewing of the T cell repertoire to a memory rather than naive T cell phenotype with age. The diminished T cell repertoire however, may not cope if the individual encounters new pathogens, possibly accounting for the rise in immunodeficiency in the aged. Obviously, there is a need to re-establish the T cell pool in immunocompromised individuals. Castration

allows the thymus to repopulate the periphery through significantly increasing the production of naive T cells.

In the periphery, T cell numbers remained at a constant level as evidenced in the B:T cell ratios of spleen and lymph nodes, presumably due to peripheral homeostasis (Mackall *et al.*, 1995; Berzins *et al.*, 1998). However, disruption of cellular composition in the periphery was evident with the aged thymus showing a significant decrease in CD4:CD8 ratios from 2:1 in the young adult to 1:1 in the 2 year mouse, possibly indicative of the more susceptible nature of $CD4^+$ T cells to age or an increase in production of $CD8^+$ T cells from extrathymic sources. By 2 weeks post-castration, this ratio has been normalised, again reflecting the immediate response of the immune system to surgical castration.

The above findings have shown firstly that the aged thymus is capable of functioning in a nature equivalent to the pre-pubertal thymus. In this respect, T cell numbers are significantly decreased but the ability of thymocytes to differentiate is not disturbed. Their overall ability to proliferate and eventually migrate to the periphery is again not influenced by the age-associated atrophy of the thymus. However, two important findings were noted. Firstly, there appears to be an adverse affect on the TN cells in their ability to proliferate, correlating with findings by Aspinall (1997). This defect could be attributed to an inherent defect in the thymocytes themselves. Yet our data, and previous work has shown thymocyte differentiation, although diminished, still occurs and stem cell entry from the BM is also not affected with age (Hirokawa, 1998; Mackall and Gress, 1997). This implicates the thymic stroma as the target for sex steroid action and consequently abnormal regulation of this precursor subset of T cells. Secondly, the $CD8^+$ T cells were significantly diminished in their proliferative capacity with age and, following castration, a significantly increased proportion of $CD8^+$ T cells proliferated as compared to the 2 month mouse. The proliferation of mature T cells is thought to be a final step before migration (Suda and Zlotnik, 1992), such that a significant decrease in $CD8^+$ proliferation would indicate a decrease in their migrational potential. This hypothesis is supported by our finding that the ratio of CD4:CD8 T cells in RTEs increased with age, indicative of a decrease in CD8 T cells migrating. Alternatively, if the thymic epithelium is providing the key factor for the CD8 T cell maintenance, whether a lymphostromal molecule or cytokine

influence, this factor may be disturbed with increased sex-steroid production. By removing the influence of sex-steroids, the CD8 T cell population can again proliferate optimally. Thus, it was necessary to determine, in detail, the status of thymic epithelial cells pre- and post-castration.

The cortex appears to 'collapse' with age due to lack of thymocytes available to expand the network of epithelium. The most dramatic change in thymic epithelium post-castration was the increased network of cortical epithelium detected by MTS 44, illustrating the significant rise in thymocyte numbers. At 2 weeks post-castration, KNAs are abundant and appear to accommodate proliferating thymocytes indicating that thymocyte development is occurring at a rate higher than the epithelium can cope with. The increase in cortical epithelium appears to be due to stretching of the thymic architecture rather than proliferation of this subtype since no proliferation of the epithelium was noted with BrdU staining by immunofluorescence.

Medullary epithelium is not as susceptible to age influences most likely due to the lesser number of T cells accumulating in this area (>95% of thymocytes are lost at the DP stage due to selection events). However, the aged thymus shows severe epithelial cell disruption distinguished by a lack of distinction of the cortico-medullary junction with the medullary epithelium incorporating into the cortical epithelium. By 2 weeks post-castration, the medullary epithelium, as detected by MTS 10 staining is re-organised to some extent, however, subpockets are still present within the cortical epithelium. By 4 weeks post-castration, the cortical and medullary epithelium is completely reorganised with a distinct cortico-medullary junction similar to the young adult thymus.

Subtle changes were also observed following castration, most evident in the decreased expression of MHC class II and blood-thymus barrier antigens when compared to the pre-castrate thymus. MHCII (detected by MTS6) is increased in expression in the aged thymus possibly relating to a decrease in control by the developing thymocytes due to their diminished numbers. Alternatively, it may simply be due to lack of masking by the thymocytes, illustrated also in the post-irradiation thymus (Randle and Boyd, 1992) which is depleted of the DP thymocytes. Once thymocyte numbers are increased following castration, the antigen binding sites are again blocked by the accumulation of thymocytes thus decreasing detection by

immunofluorescence. The antigens detecting the blood-thymus barrier (MTS12, 15 and 16) are again increased in the aged thymus and also revert to the expression in the young adult thymus post-castration. Lack of masking by thymocytes and the close proximity of the antigens due to thymic atrophy may explain this increase in expression. Alternatively, the developing thymocytes may provide the necessary control mechanisms over the expression of these antigens thus when these are depleted, expression is not controlled. The primordial epithelial antigens detected by MTS 20 and MTS 24 are increased in expression in the aged thymus but revert to subpockets of epithelium at the cortico-medullary junction post-castration. This indicates a lack of signals for this epithelial precursor subtype to differentiate in the aged mouse. Removing the block placed by the sex-steroids, these antigens can differentiate to express cortical epithelial antigens.

The above findings indicate a defect in the thymic epithelium rendering it incapable of providing the developing thymocytes with the necessary stimulus for development. However, the symbiotic nature of the thymic epithelium and thymocytes makes it difficult to ascertain the exact pathway of destruction by the sex steroid influences. The medullary epithelium requires cortical T cells for its proper development and maintenance. Thus, if this population is diminished, the medullary thymocytes may not receive adequate signals for development. This particularly seems to affect the $CD8^+$ population. $IRF^{-/-}$ mice show a decreased number of $CD8^+$ T cells. It would therefore, be interesting to determine the proliferative capacity of these cells.

The defect in proliferation of the TN1 subset which was observed indicates that loss of cortical epithelium affects thymocyte development at the crucial stage of TCR gene rearrangement whereby the cortical epithelium provides factors such as IL-7 and SCF necessary for thymopoiesis (Godfrey and Zlotnik, 1990; Aspinall, 1997). Indeed, $IL\text{-}7^{-/-}$ and $IL\text{-}7R^{-/-}$ mice show similar thymic morphology to that seen in aged mice (Wiles *et al.*, 1992; Zlotnik and Moore, 1995; von Freeden-Jeffry, 1995). Further work is necessary to determine the changes in IL-7 and IL-7R with age.

In conclusion, the aged thymus still maintains its functional capacity, however, the thymocytes that develop in the aged mouse are not under the stringent control by thymic epithelial cells as seen in the normal young mouse due to the lack of structural integrity of the thymic

microenvironment. Thus the proliferation, differentiation and migration of these cells will not be under optimal regulation and may result in the increased release of autoreactive/immunodysfunctional T cells in the periphery. The defects within both the TN and particularly, $CD8^+$ populations, may result in the changes seen within the peripheral T cell pool with age. In addition, we have described in detail, the effects of castration on thymic epithelial cell development and reorganisation. The mechanisms underlying thymic atrophy utilising steroid receptor binding assays and the role of thymic epithelial subsets in thymus regeneration post-castration are currently under study. Restoration of thymus function by castration will provide an essential means for regenerating the peripheral T cell pool and thus in re-establishing immunity in immunosuppressed individuals.

The impact of castration on thymic structure and T cell production was investigated in animal models of immunodepletion. Specifically, Example 2 examined the effect of castration on the recovery of the immune system after sublethal irradiation and cyclophosphamide treatment. These forms of immunodepletion act to inhibit DNA synthesis and therefore target rapidly dividing cells. In the thymus these cells are predominantly immature cortical thymocytes, however all subsets are effected (Fredrickson and Basch 1994). In normal healthy aged mice, the qualitative and quantitative deviations in peripheral T cells seldom lead to pathological states. However, major problems arise following severe depletion of T cells because of the reduced capacity of the thymus for T cell regeneration. Such insults occur in HIV/AIDS, and particularly following chemotherapy and radiotherapy in cancer treatment (Mackall *et al.* 1995).

In both sublethally irradiated and cyclophosphamide treated mice, castration markedly enhanced thymic regeneration. Castration was carried out on the same day as and seven days prior to immunodepletion in order to appraise the effect of the predominantly corticosteroid induced, stress response to surgical castration on thymic regeneration. Although increases in thymus cellularity and architecture were seen as early as one week after immunodepletion, the major differences were observed two weeks after castration. This was the case whether castration was performed on the same day or one week prior to immunodepletion.

Immunohistology demonstrated that in all instances, two weeks after castration the thymic architecture appeared phenotypically normal, while

that of noncastrated mice was disorganised. Pan epithelial markers demonstrated that immunodepletion caused a collapse in cortical epithelium and a general disruption of thymic architecture in the thymii of noncastrated mice. Medullary markers supported this finding. Interestingly, one of the first features of castration-induced thymic regeneration was a marked upregulation in the extracellular matrix, identified by MTS 16.

Flow cytometry analysis data illustrated a significant increase in the number of cells in all thymocyte subsets in castrated mice, corresponding with the immunofluorescence. At each time point, there was a synchronous increase in all CD4, CD8 and αβ-TCR - defined subsets following immunodepletion and castration. This is an unusual but consistent result, since T cell development is a progressive process it was expected that there would be an initial increase in precursor cells (contained within the $CD4^-CD8^-$ gate) and this may have occurred before the first time point. Moreover, since precursors represent a very small proportion of total thymocytes, a shift in their number may not have been detectable. The effects of castration on other cells, including macrophages and granulocytes were also analysed. In general there was little alteration in macrophage and granulocyte numbers within the thymus.

In both irradiation and cyclophosphamide models of immunodepletion thymocyte numbers peaked at every two weeks and decreased four weeks after treatment. Almost immediately after irradiation or chemotherapy, thymus weight and cellularity decreased dramatically and approximately 5 days later the first phase of thymic regeneration begun. The first wave of reconstitution (days 5-14) was brought about by the proliferation of radioresistant thymocytes (predominantly double negatives) which gave rise to all thymocyte subsets (Penit and Ezine 1989). The second decrease, observed between days 16 and 22 was due to the limited proliferative ability of the radioresistant cells coupled with a decreased production of thymic precursors by the bone marrow (also effected by irradiation). The second regenerative phase was due to the replenishment of the thymus with bone marrow derived precursors (Huiskamp *et al.* 1983).

It is important to note that in adult mice the development from a HSC to a mature T cell takes approximately 28 days (Shortman *et al.* 1990). Therefore, it is not surprising that little change was seen in peripheral T cells up to four weeks after treatment. The periphery would be supported by some

thymic export, but the majority of the T cells found in the periphery up to four weeks after treatment would be expected to be proliferating cyclophosphamide or irradiation resistant clones expanding in the absence of depleted cells. Several long term changes in the periphery would be expected post-castration including, most importantly, a diversification of the TCR repertoire due to an increase in thymic export. Castration did not effect the peripheral recovery of other leukocytes, including B cells, macrophages and granulocytes.

Example 4 shows the influence of castration on sygeneic and congenic bone marrow transplantation. Starzl *et al.* (1992) reported that microchimeras evident in lymphoid and nonlymphoid tissue were a good prognostic indicator for allograft transplantation. That is it was postulated that they were necessary for the induction of tolerance to the graft (Starzl *et al.* 1992). Donor-derived dendritic cells were present in these chimeras and were thought to play an integral role in the avoidance of graft rejection (Thomson and Lu 1999). Dendritic cells are known to be key players in the negative selection processes of thymus and if donor-derived dendritic cells were present in the recipient thymus, graft reactive T cells may be deleted.

In order to determine if castration would enable increased chimera formation, a study was performed using syngeneic foetal liver transplantation. The results showed an enhanced regeneration of thymii of castrated mice. These trends were again seen when the experiments were repeated using congenic (Ly5) mice. Due to the presence of congenic markers, it was possible to assess the chimeric status of the mice. As early as two weeks after foetal liver reconstitution there were donor-derived dendritic cells detectable in the thymus, the number in castrated mice being four-fold higher than that in noncastrated mice. Four weeks after reconstitution the noncastrated mice did not appear to be reconstituted with donor derived cells, suggesting that castration may in fact increase the probability of chimera formation. Given that castration not only increases thymic regeneration after lethal irradiation and foetal liver reconstitution and that it also increases the number of donor-derived dendritic cells in the thymus, along-side stem cell transplantation this approach increases the probability of graft acceptance.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for modifying T-cell population makeup or increasing the number of T-cells in a subject having depressed or abnormal T-cell population or function, the method comprising disrupting sex steroid signalling to the thymus in the subject.

2. The method according to claim 1, wherein the modification of T-cell population makeup is characterized by an alteration in the nature and/or ratio of T cell subsets defined functionally and/or by expression of characteristic molecules, wherein the characteristic molecules are selected from the group consisting of: the T cell receptor, CD4, CD8, CD3, CD25, CD28, CD44, CD62L and CD69.

3. The method according claim 1 or claim 2, wherein increasing the number of T-cells in a subject results in a relative increase in T cell numbers when compared to other lymphoid cells.

4. The method according to claims 3, wherein the other lymphoid cells are B cells.

5. The method according to any one of claims 1 to 4, wherein the subject having a depressed or abnormal T-cell population or function is suffering from a condition selected from the group consisting of: cancer, human immunodeficiency virus infection, an autoimmune disease, a hypersensitivity disease or endometriosis.

6. The method according to claim 5, wherein the cancer sufferer has undergone chemotherapy and/or radiation therapy and/or bone marrow transplantation.

7. The method according to claim 5, wherein the subject with the human immunodeficiency virus infection has AIDS.

8. The method according to any one of claims 1 to 7, wherein the subject is post-pubertal.

9. A method of treating a subject suffering from an autoimmune disease, the method comprising;

ablating the resident T cell population of the subject, and

disrupting sex steroid signalling to the thymus.

10. The method according to claim 9, wherein the method further comprises subjecting the individual to a bone marrow transplant.

11. The method according to claim 9 or claim 10, wherein the T cell population is ablated by exposing the individual to chemotherapy or irradiation.

12. A method for enhancing an immune response to an antigen in a subject, the method comprising the steps of:

disrupting sex steroid signalling to the thymus in the subject, and

administering the antigen.

13. The method of claim 12, wherein the antigen is derived from an infectious agent or a tumour cell.

14. The method of claim 13, wherein the subject has cancer.

15. The method of claim 13, wherein the subject has an infection.

16. The method according to any one of claims 12 to 15, wherein the antigen is mixed with an adjuvant before administration.

17. A method of decreasing host-vs-graft reaction in a subject following transplantation of an organ, the method comprising the steps of:

ablating T-cells in the subject;

disrupting sex steroid signalling to the thymus of the subject; and

transplanting the organ from the donor to the subject.

18. The method according to claim 17, further comprising the step of transplanting bone marrow to the subject from a donor.

19. The method according to any one of claims 1 to 18, wherein sex steroid signalling to the thymus is disrupted by inhibiting sex steroid production or by blocking a sex steroid receptor(s) within the thymus.

20. The method according to claim 19, wherein inhibition of sex steroid production is achieved by either castration or administration of a sex steroid analogue(s).

21. The method according to claim 20, wherein the sex steroid analogue is selected from the group consisting of: eulexin, goserelin, leuprolide, dioxalan derivatives such as triptorelin, meterelin, buserelin, histrelin, nafarelin, lutrelin, leuprorelin, and luteinizing hormone-releasing hormone analogues.

22. The method according to claim 21, wherein the sex steroid analogue is an analogue of luteinizing hormone-releasing hormone.

23. The method according to claim 22, wherein the luteinizing hormone-releasing hormone analogue is deslorelin.

24. The method according to any one of claims 1 to 23, wherein the sex steroid analogue(s) is administered by a sustained peptide-release formulation.

25. A composition for enhancing an immune response to an antigen in a subject, the composition comprising an adjuvant, the antigen, and an analogue of luteinizing hormone-releasing hormone.

26. The method according to any one of claims 1 to 24, wherein the subject is a mammal.

27. The method according to claim 26, wherein the mammal is a human.

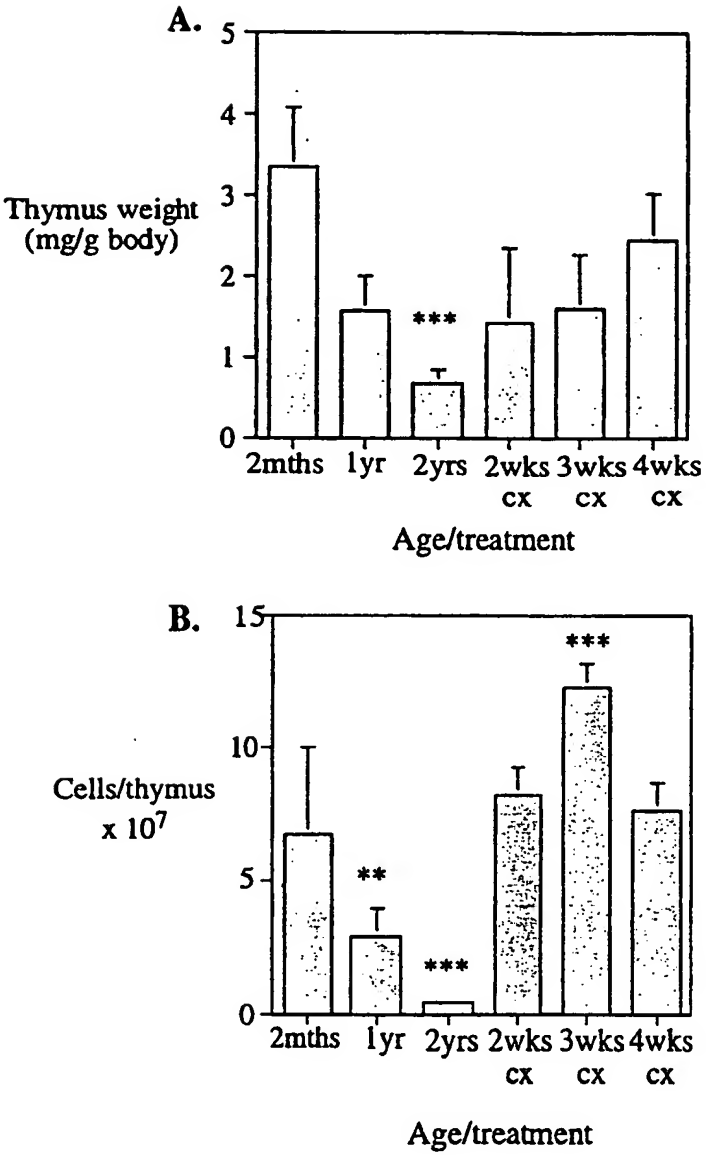

A.
Thymus weight
(mg/g body)
0
1
2
3
4
5
***
2mths
1yr
2yrs
2wks cx
3wks cx
4wks cx
Age/treatment
B.
Cells/thymus
x 10^7
0
5
10
15
**
***
***
2mths
1yr
2yrs
2wks cx
3wks cx
4wks cx
Age/treatment

**Figure 1**

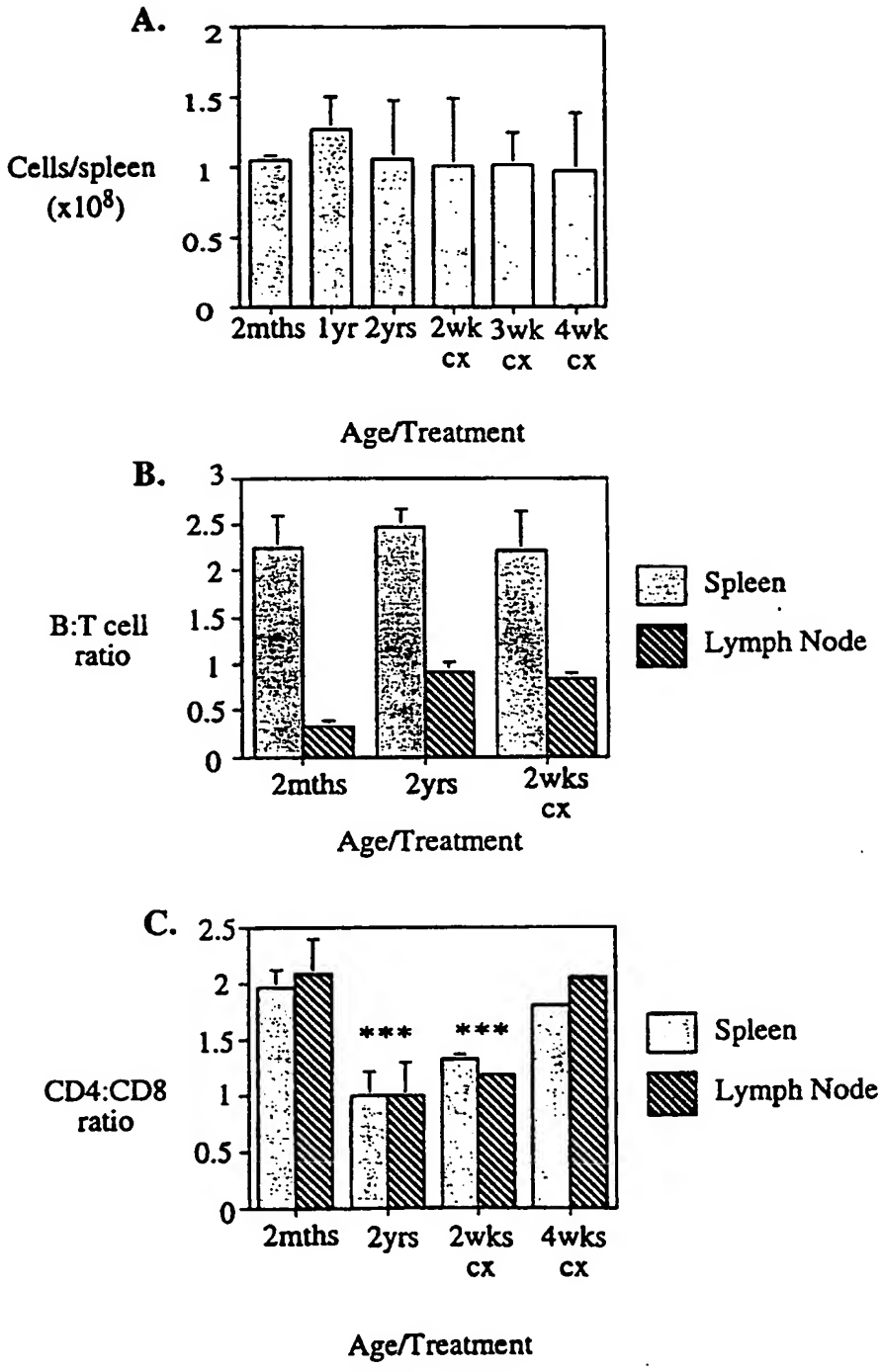

A.
Cells/spleen
(x10^8)
2
1.5
1
0.5
0
2mths
1yr
2yrs
2wk cx
3wk cx
4wk cx
Age/Treatment
B.
B:T cell ratio
3
2.5
2
1.5
1
0.5
0
2mths
2yrs
2wks cx
Spleen
Lymph Node
Age/Treatment
C.
CD4:CD8 ratio
2.5
2
1.5
1
0.5
0
***
***
2mths
2yrs
2wks cx
4wks cx
Spleen
Lymph Node
Age/Treatment

**Figure 2**

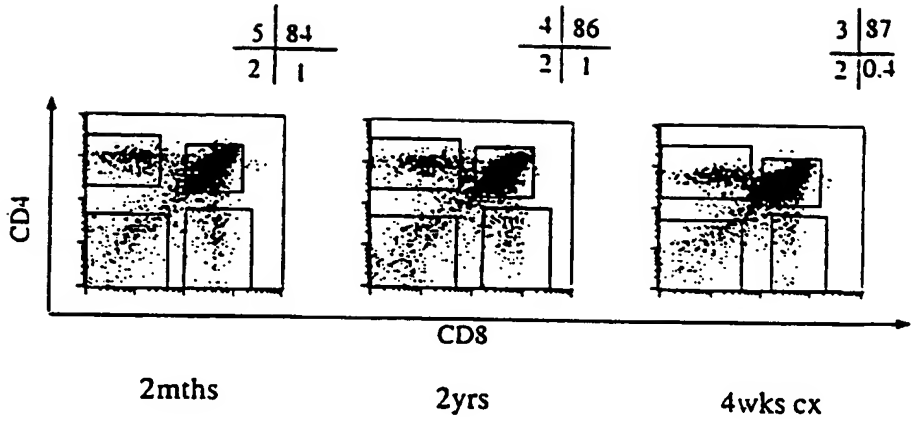



Figure 3

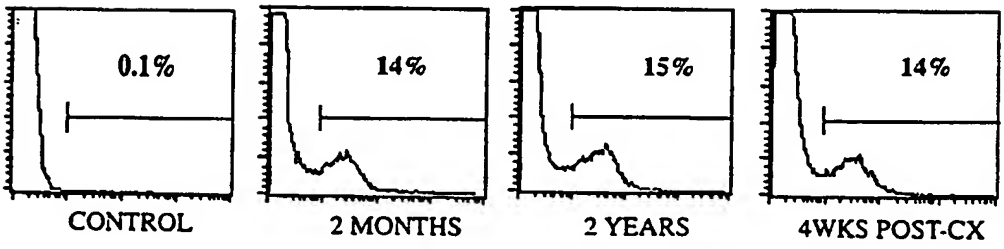



Figure 4.1

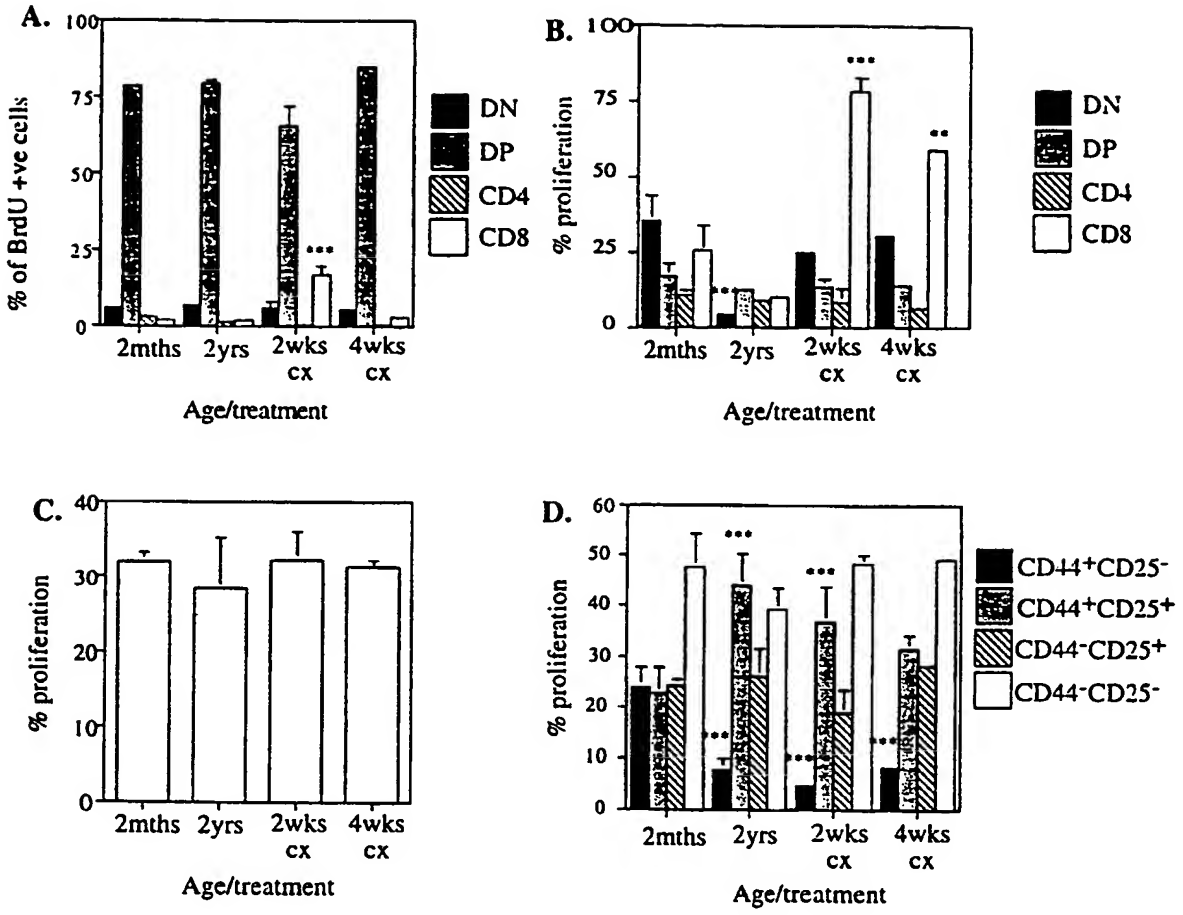

A.
% of BrdU +ve cells
100
75
50
25
0
2mths
2yrs
2wks cx
4wks cx
Age/treatment
DN
DP
CD4
CD8
B.
% proliferation
Age/treatment
C.
% proliferation
Age/treatment
D.
% proliferation
Age/treatment
CD44+CD25-
CD44+CD25+
CD44-CD25+
CD44-CD25-

Figure 4.2

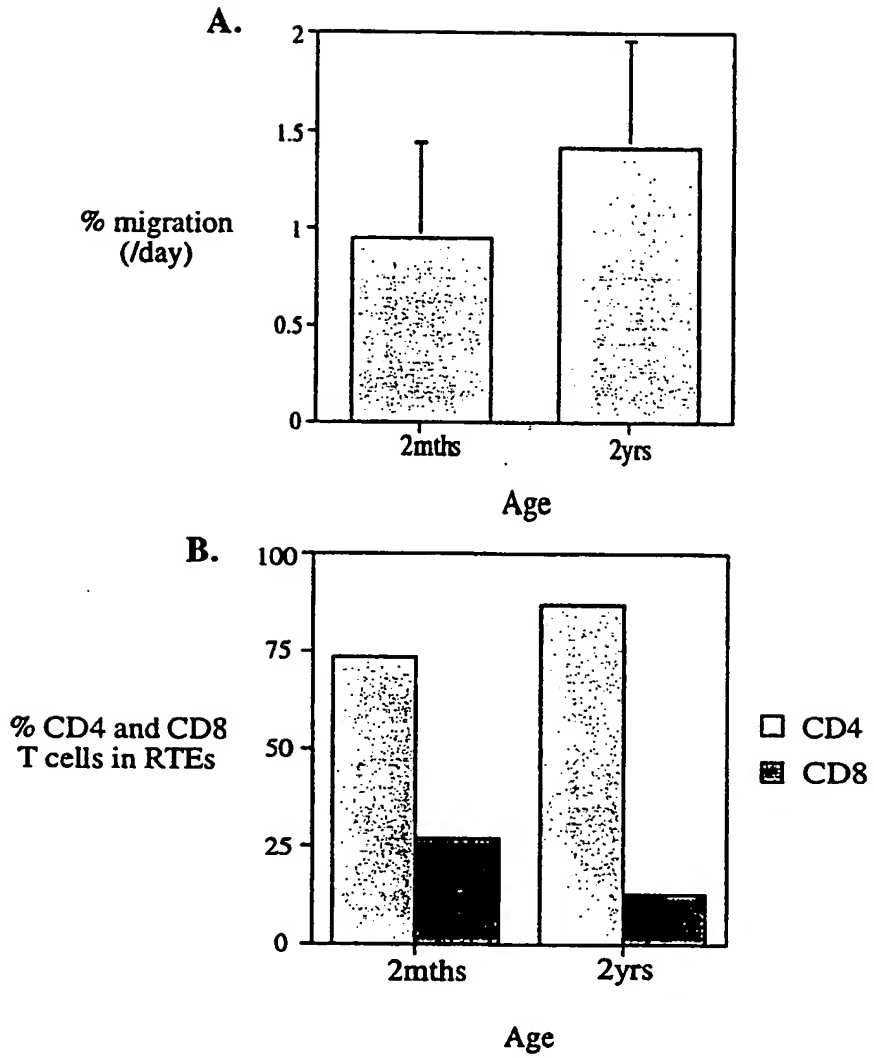

A.
% migration (/day)
2
1.5
1
0.5
0
2mths
2yrs
Age
B.
% CD4 and CD8 T cells in RTEs
100
75
50
25
0
2mths
2yrs
Age
CD4
CD8

**Figure 5**

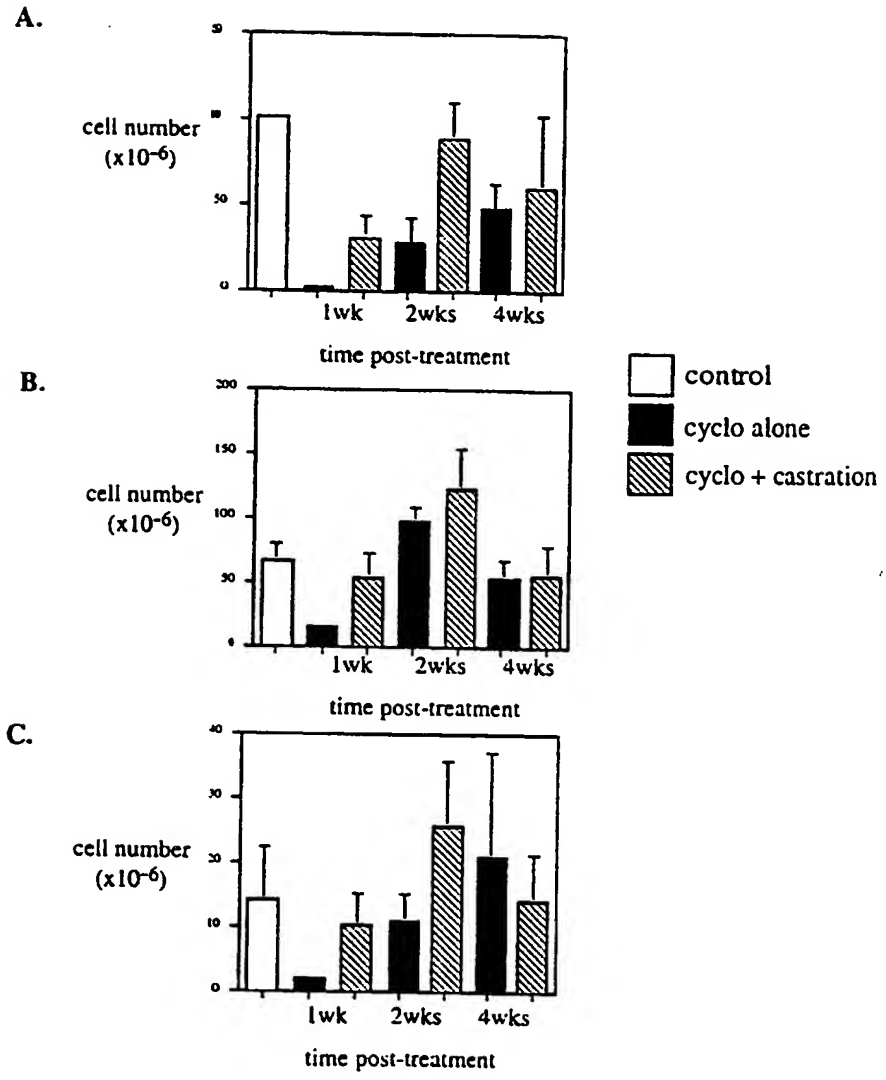

A.
cell number
(x10-6)
1wk
2wks
4wks
time post-treatment
B.
cell number
(x10-6)
1wk
2wks
4wks
time post-treatment
C.
cell number
(x10-6)
1wk
2wks
4wks
time post-treatment
control
cyclo alone
cyclo + castration

Figure 6

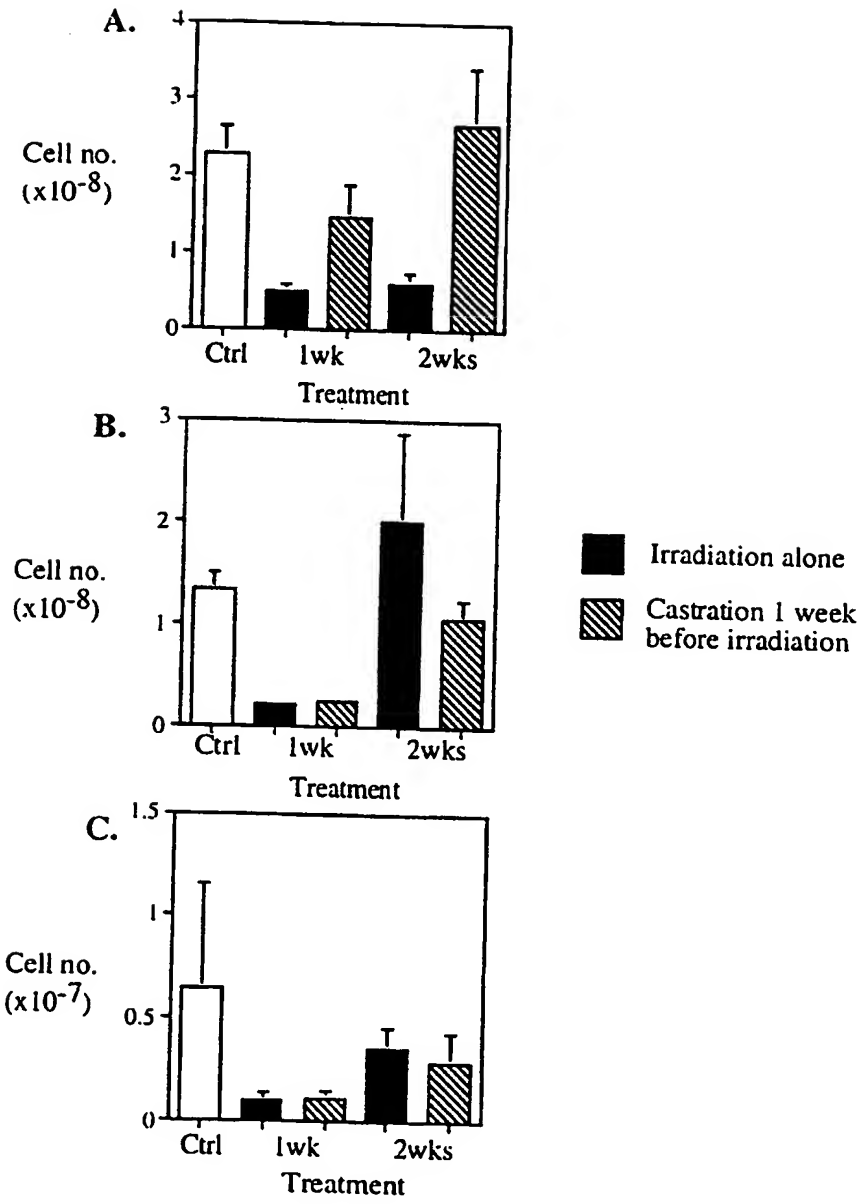

A.
Cell no.
(x10-8)
0
1
2
3
4
Ctrl
1wk
2wks
Treatment
B.
Cell no.
(x10-8)
0
1
2
3
Ctrl
1wk
2wks
Treatment
C.
Cell no.
(x10-7)
0
0.5
1
1.5
Ctrl
1wk
2wks
Treatment
Irradiation alone
Castration 1 week before irradiation

**Figure 7**

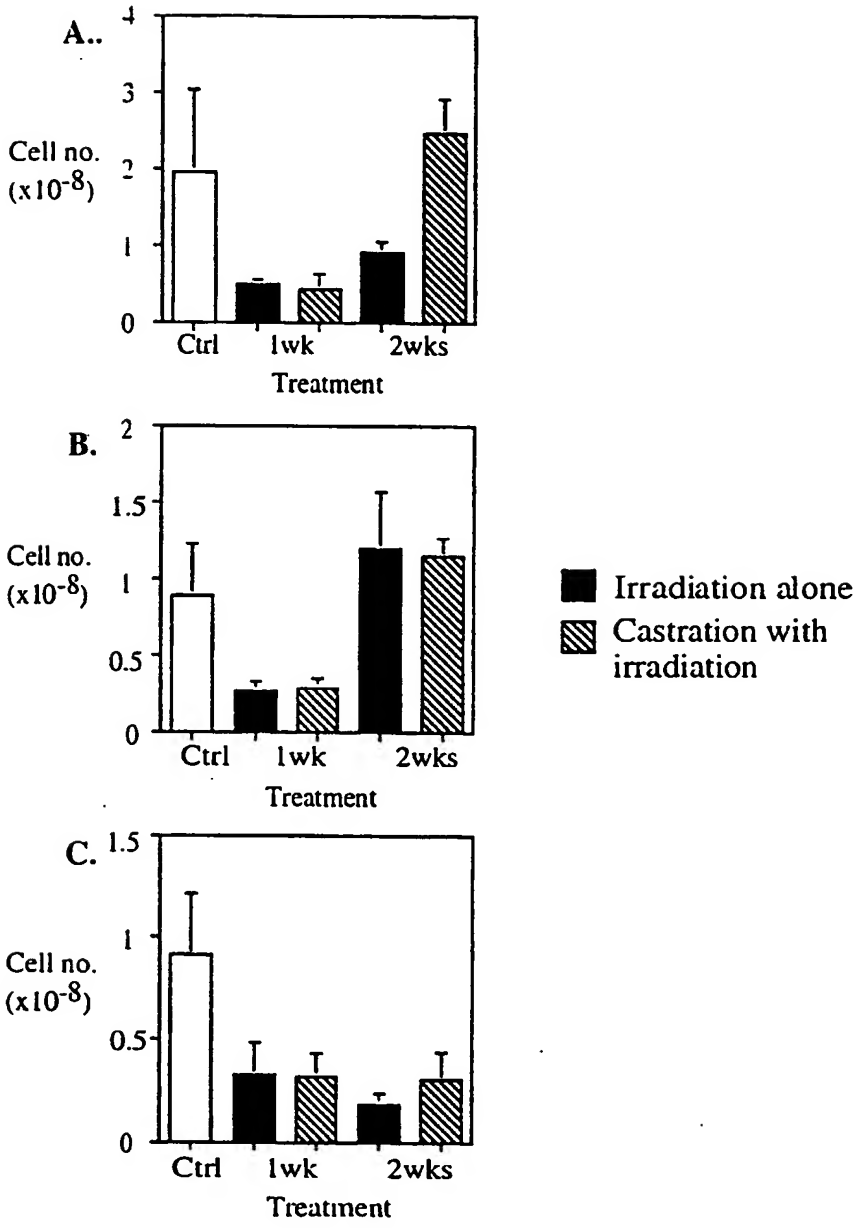

A..
Cell no. (x10-8)
4
3
2
1
0
Ctrl
1wk
2wks
Treatment
B.
Cell no. (x10-8)
2
1.5
1
0.5
0
Ctrl
1wk
2wks
Treatment
C.
Cell no. (x10-8)
1.5
1
0.5
0
Ctrl
1wk
2wks
Treatment
Irradiation alone
Castration with irradiation

Figure 8

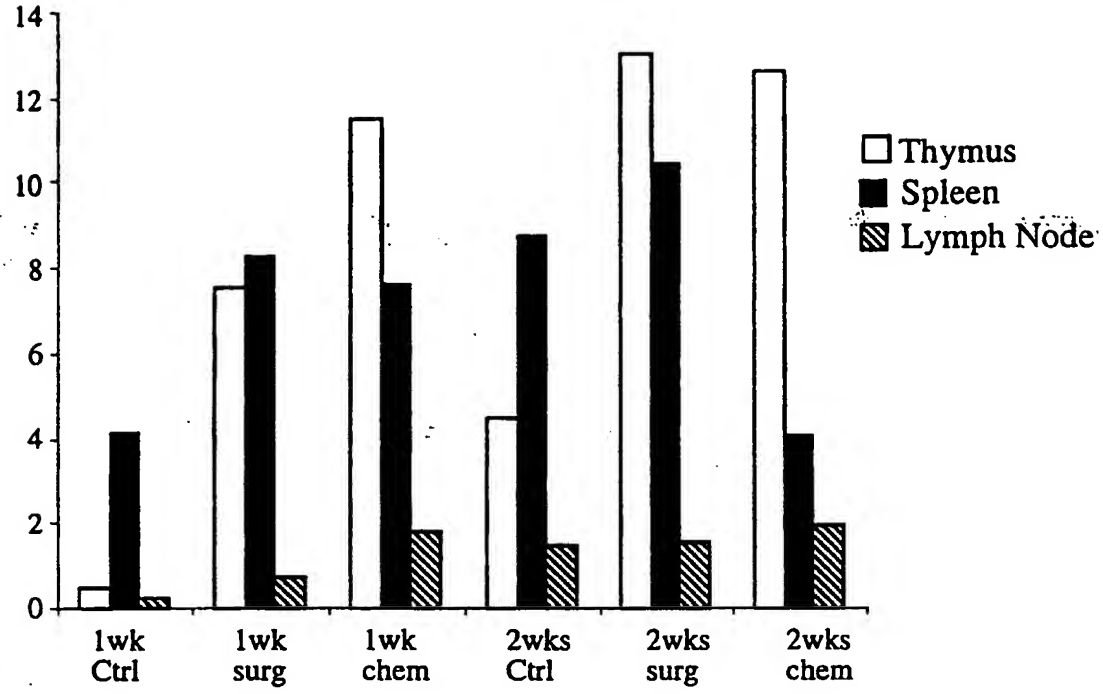

14
12
10
8
6
4
2
0
1wk Ctrl
1wk surg
1wk chem
2wks Ctrl
2wks surg
2wks chem
Thymus
Spleen
Lymph Node

**Figure 9**

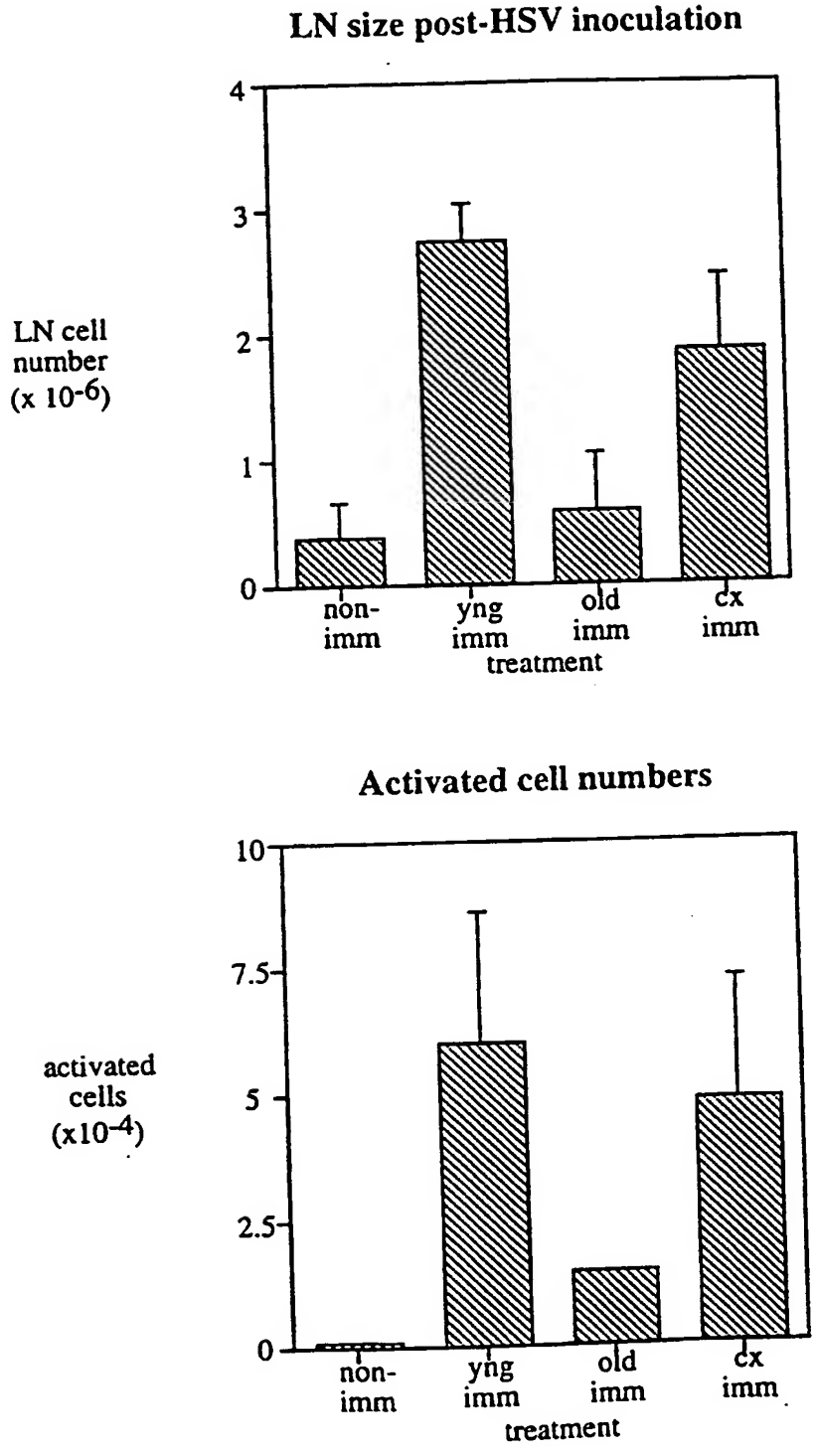

LN size post-HSV inoculation
LN cell number (x 10-6)
4
3
2
1
0
non-imm
yng imm
old imm
cx imm
treatment
Activated cell numbers
activated cells (x10-4)
10
7.5
5
2.5
0
non-imm
yng imm
old imm
cx imm
treatment

Figure 10

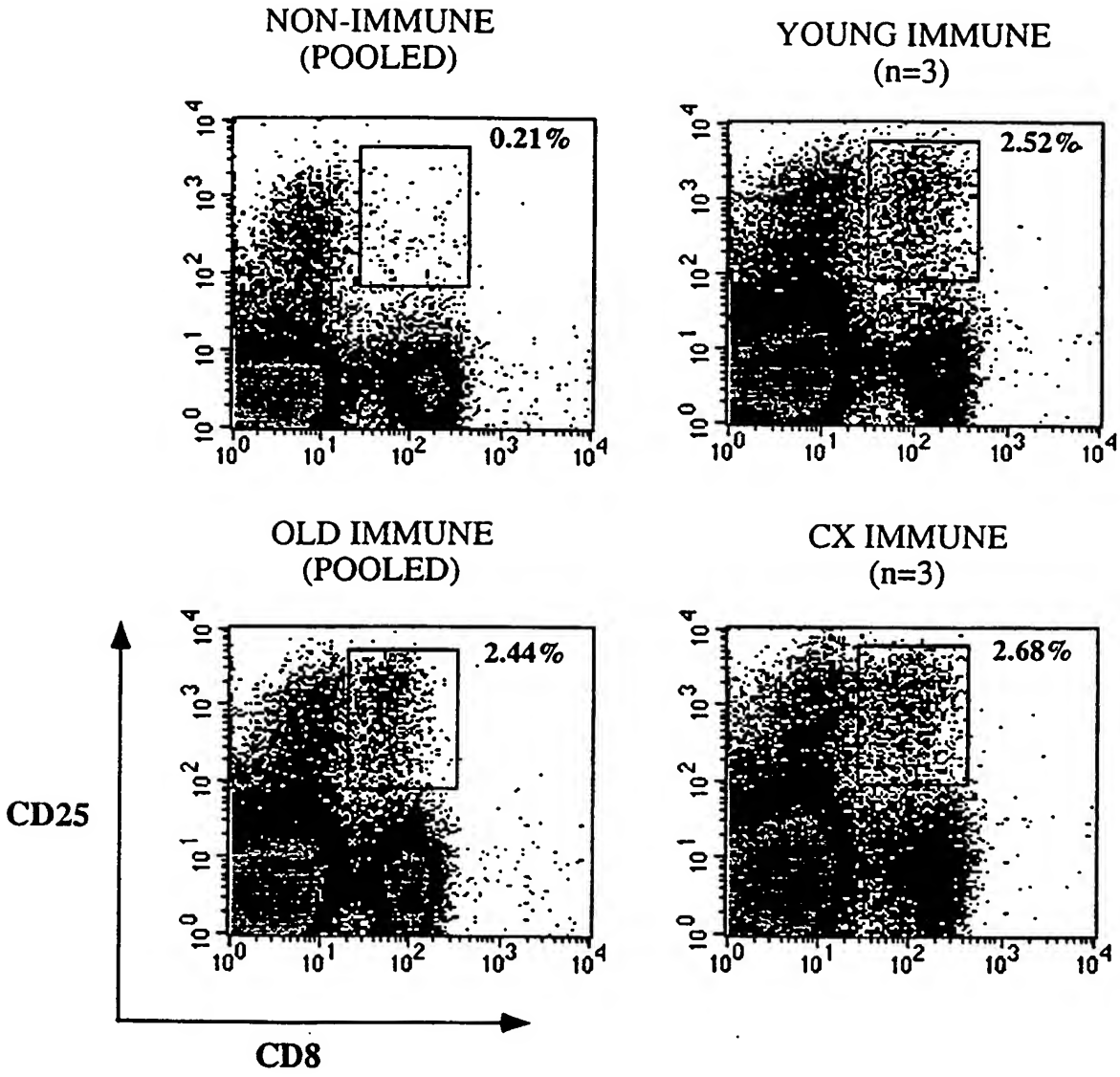

NON-IMMUNE
(POOLED)
0.21%
YOUNG IMMUNE
(n=3)
2.52%
OLD IMMUNE
(POOLED)
2.44%
CX IMMUNE
(n=3)
2.68%
CD25
CD8

Figure 11

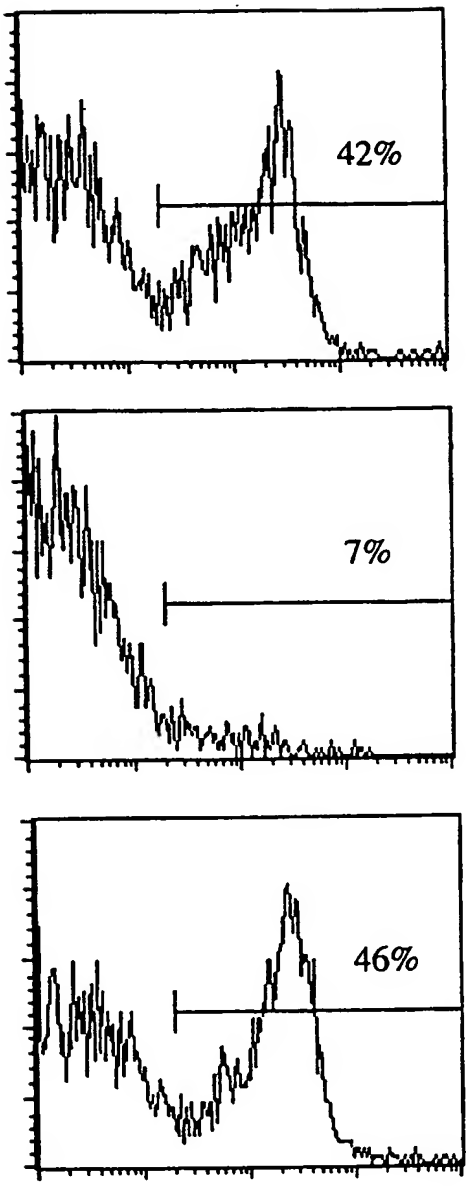



**Figure 12**

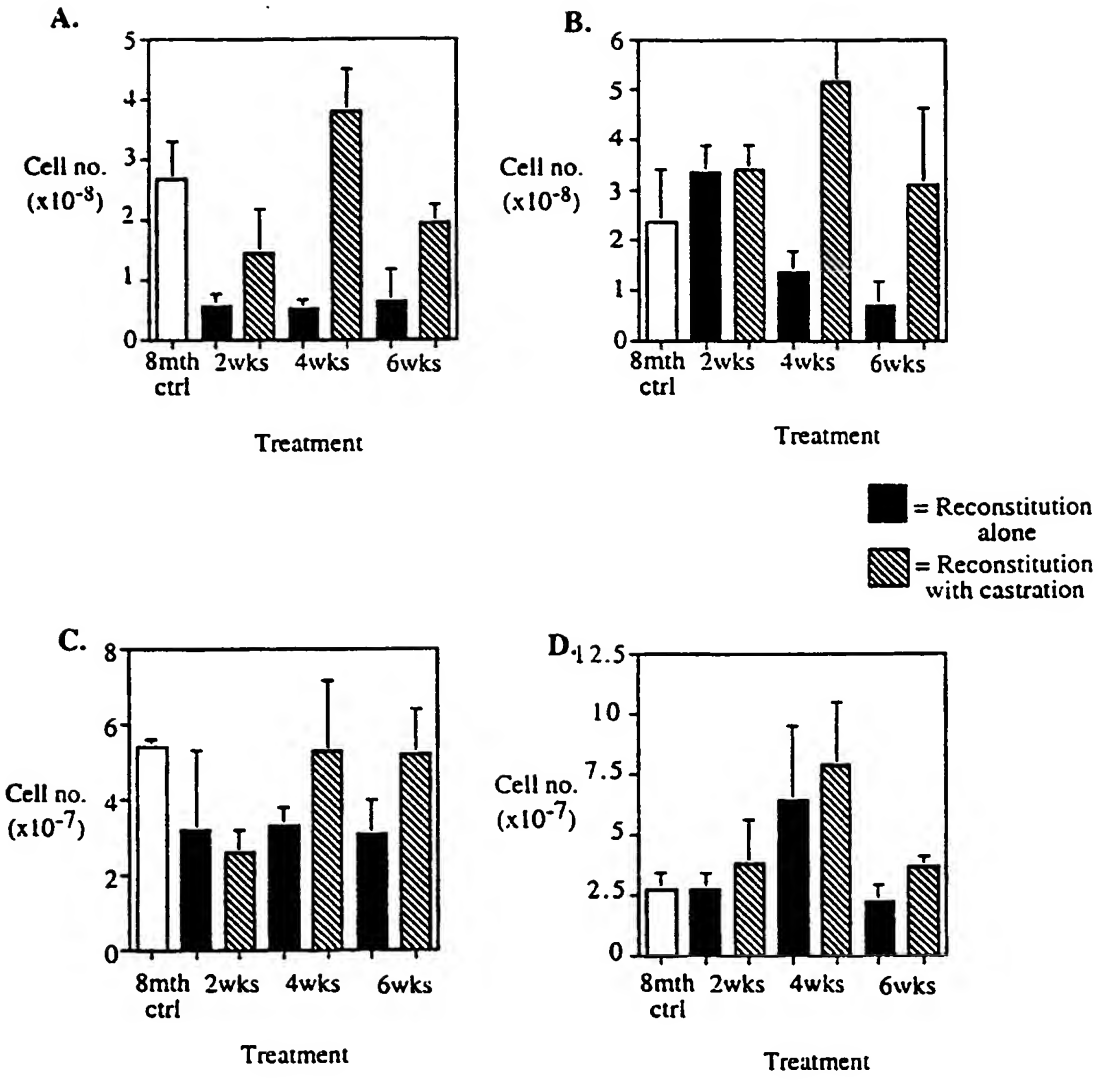

A.
Cell no. (x10-8)
8mth ctrl
2wks
4wks
6wks
Treatment
B.
Cell no. (x10-8)
Treatment
= Reconstitution alone
= Reconstitution with castration
C.
Cell no. (x10-7)
Treatment
D.
Cell no. (x10-7)
Treatment

Figure 13

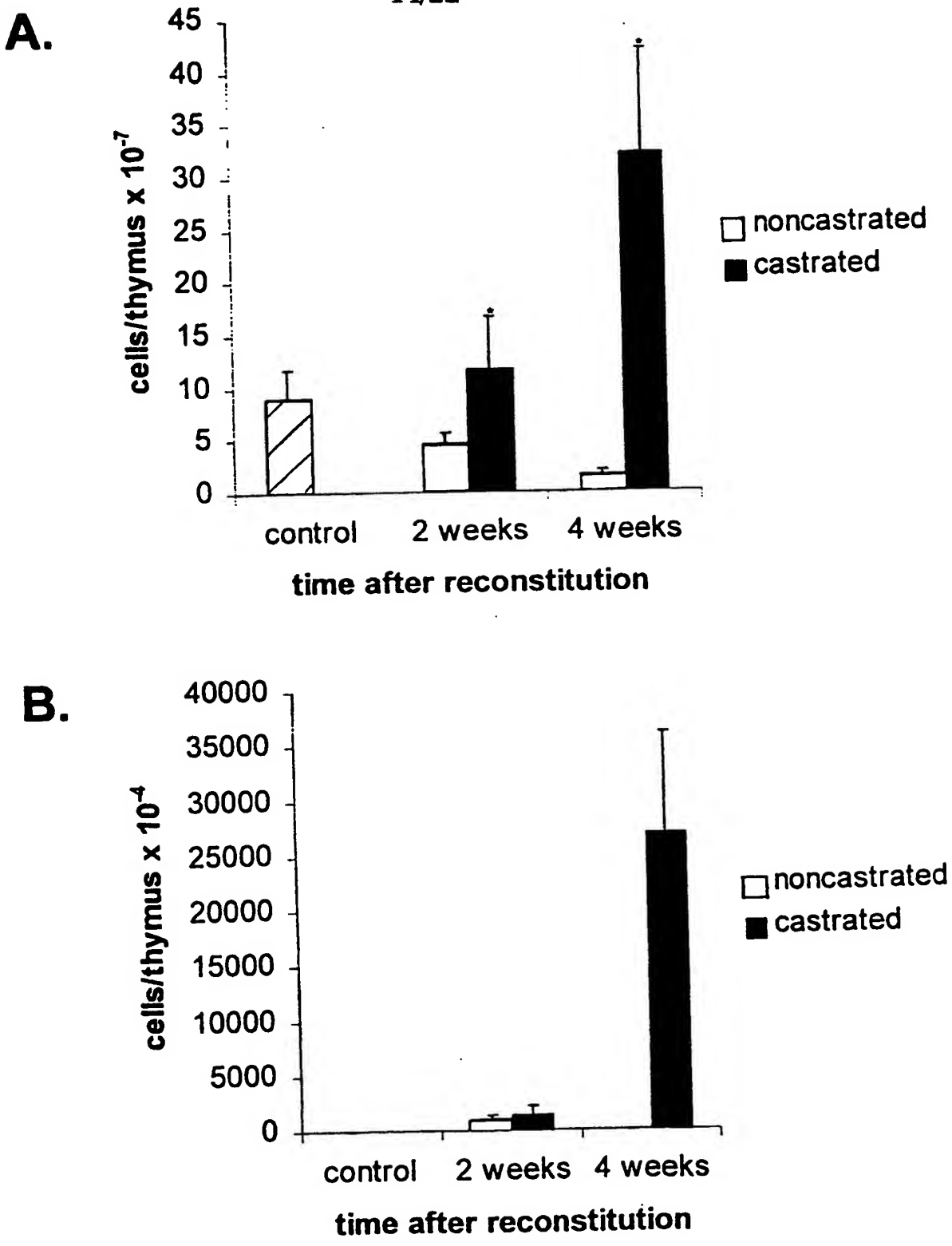

A.
45
40
35
30
25
20
15
10
5
0
cells/thymus x 10-7
noncastrated
castrated
control
2 weeks
4 weeks
time after reconstitution
B.
40000
35000
30000
25000
20000
15000
10000
5000
0
cells/thymus x 10-4
noncastrated
castrated
control
2 weeks
4 weeks
time after reconstitution

Figure 14

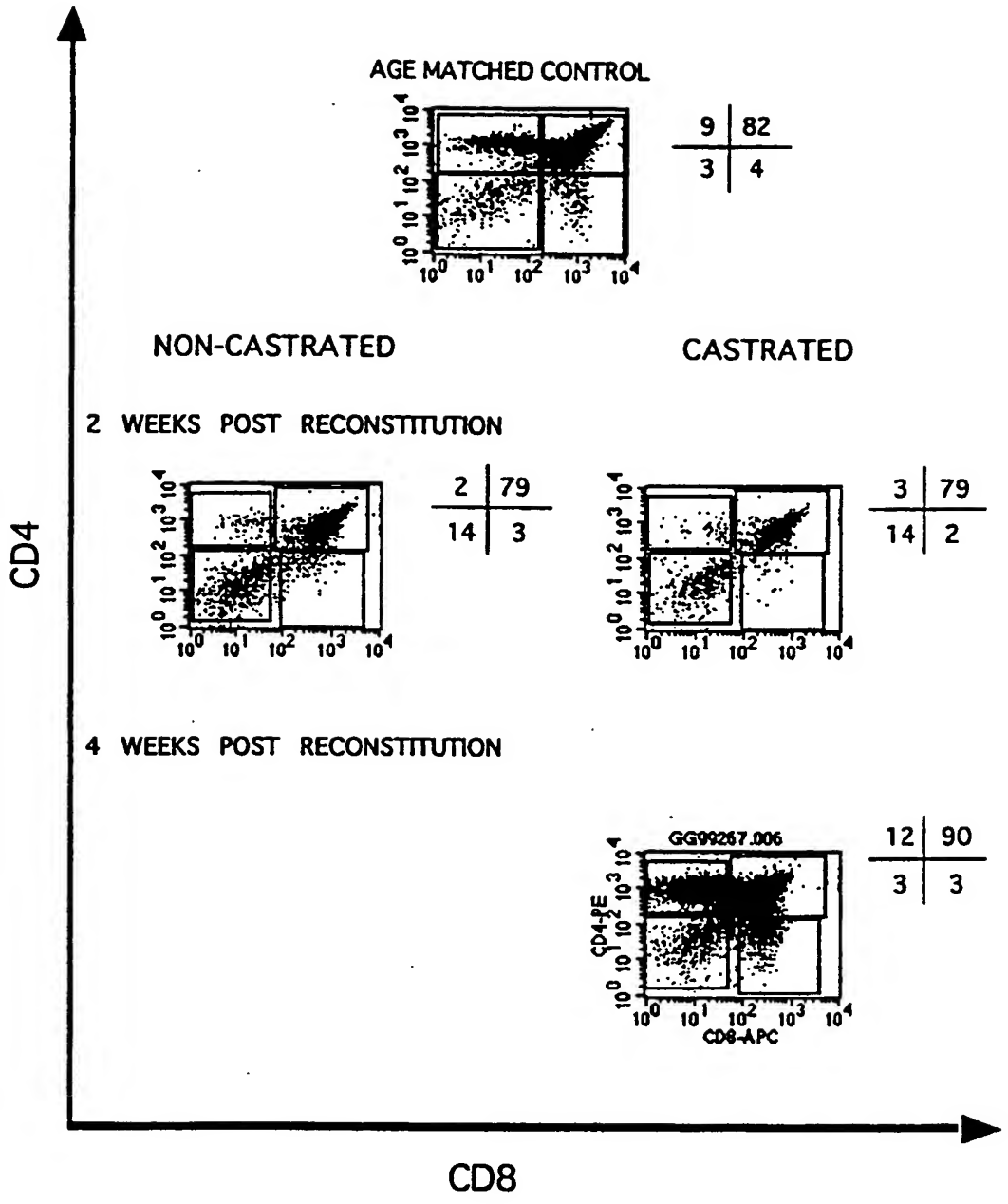
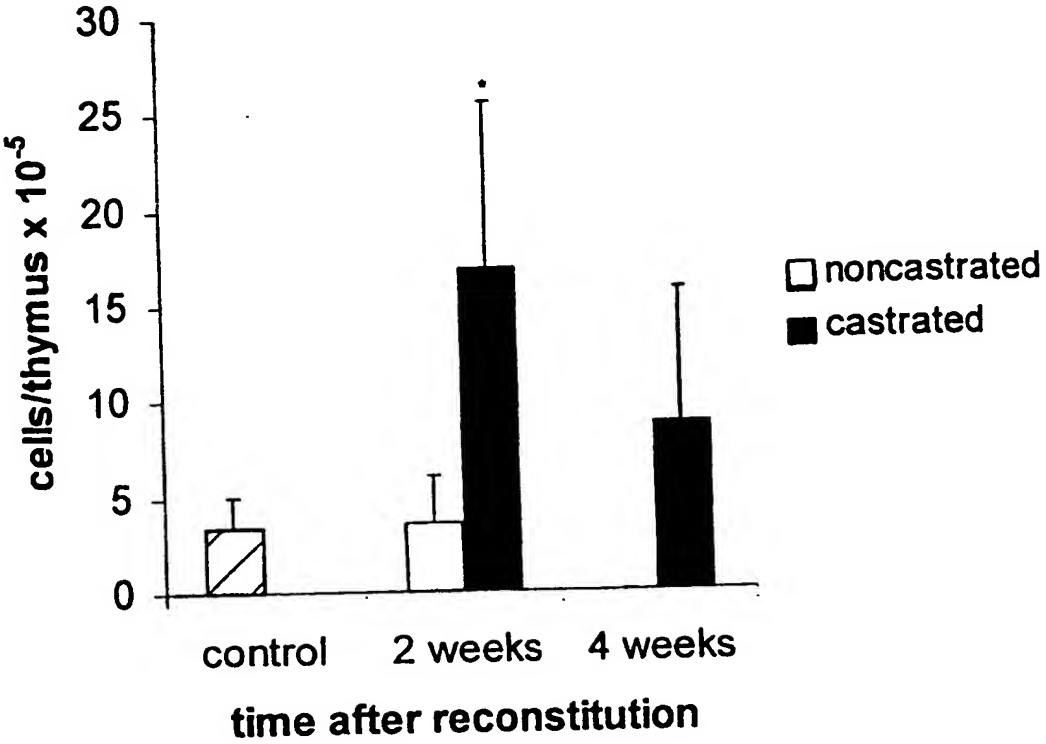

AGE MATCHED CONTROL
9 82
3 4
NON-CASTRATED
CASTRATED
2 WEEKS POST RECONSTITUTION
2 79
14 3
3 79
14 2
4 WEEKS POST RECONSTITUTION
GG99267.006
12 90
3 3
CD4-PE
CD8-APC
CD4
CD8

Figure 15

A.


30
25
20
15
10
5
0
cells/thymus x 10^-5
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated

B.

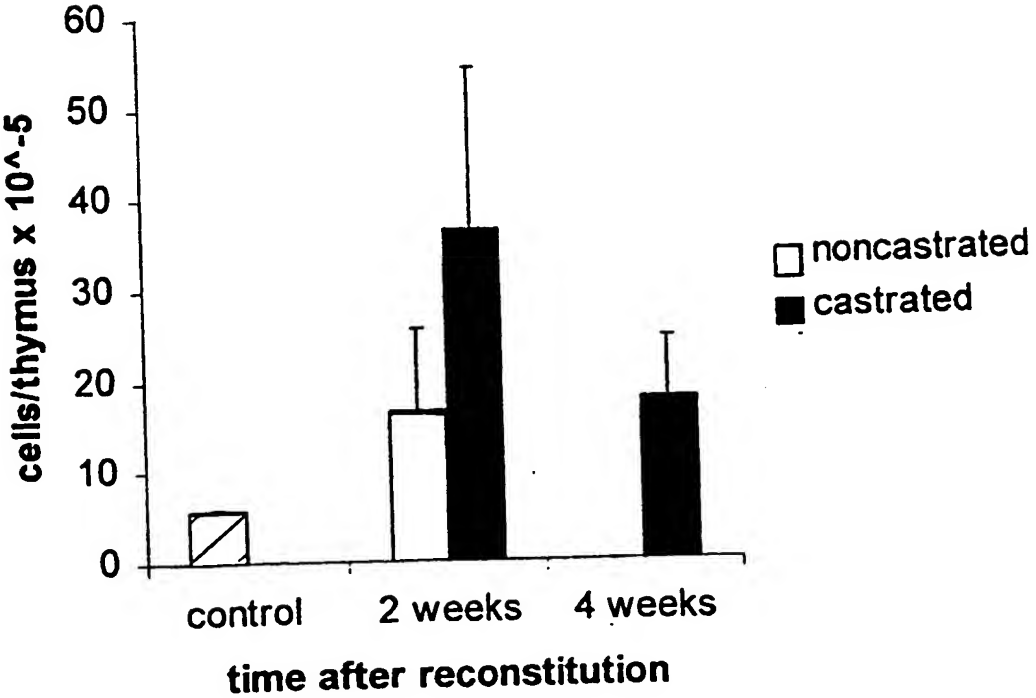

60
50
40
30
20
10
0
cells/thymus x 10^-5
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated

Figure 16

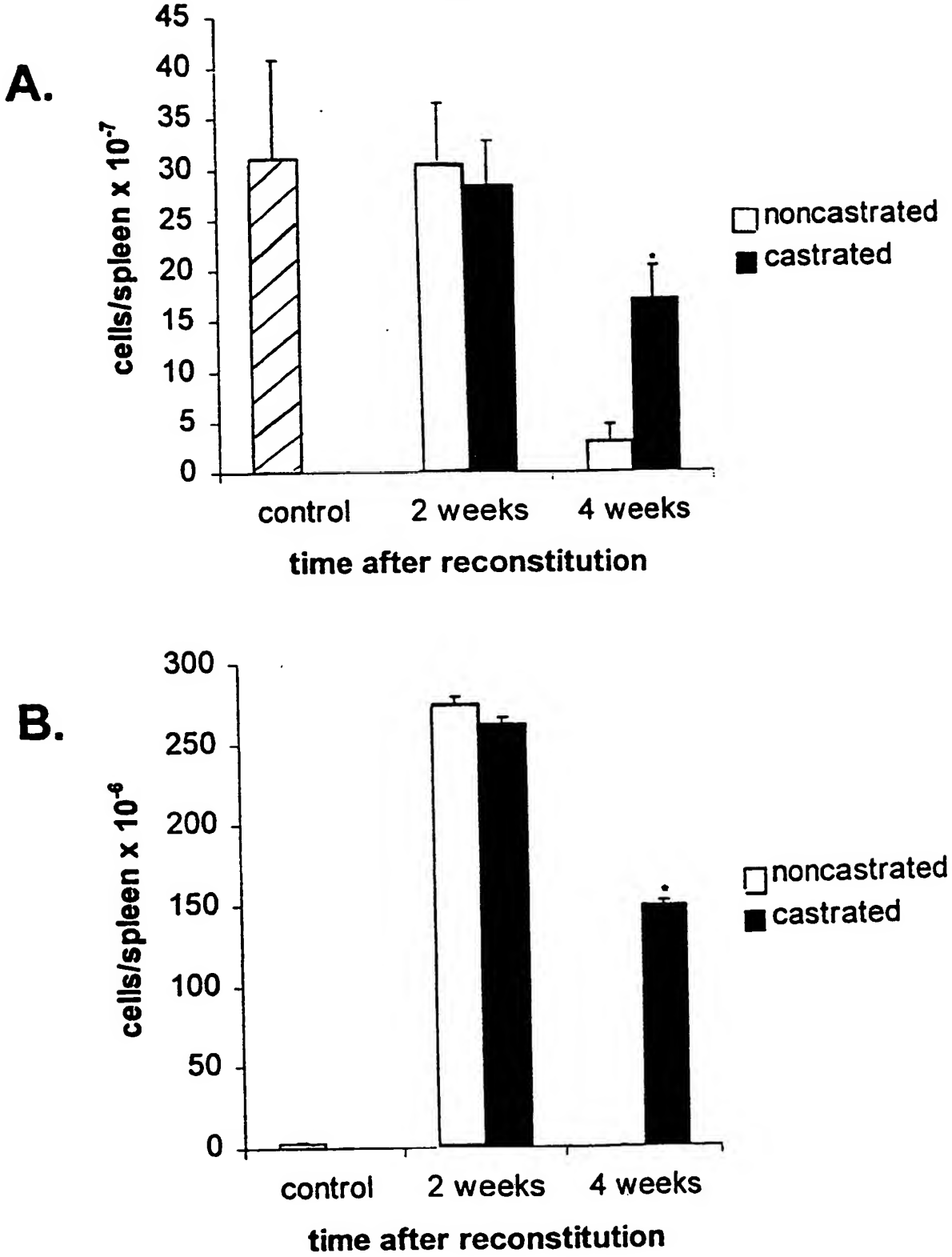

A.
45
40
35
30
25
20
15
10
5
0
cells/spleen x 10⁻⁷
noncastrated
castrated
control
2 weeks
4 weeks
time after reconstitution
B.
300
250
200
150
100
50
0
cells/spleen x 10⁻⁶
noncastrated
castrated
control
2 weeks
4 weeks
time after reconstitution

Figure 17

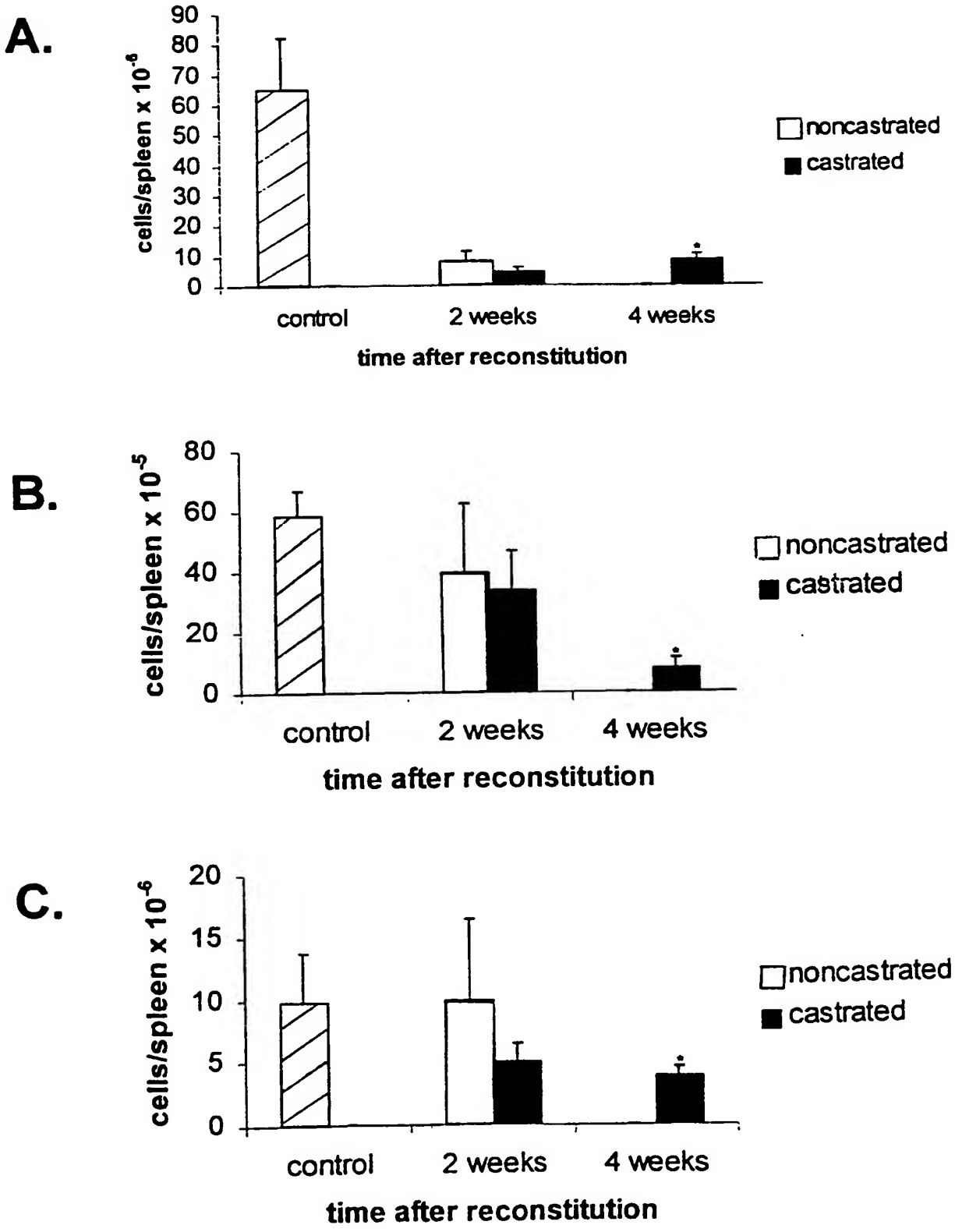
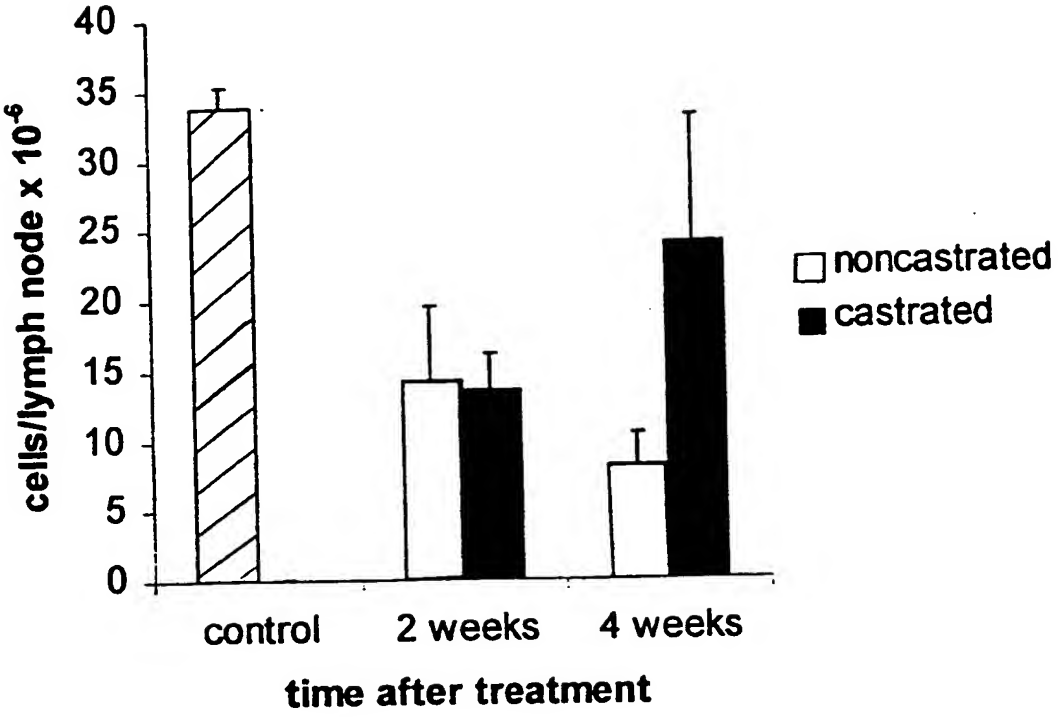

A.
cells/spleen x 10⁻⁶
90
80
70
60
50
40
30
20
10
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated
B.
cells/spleen x 10⁻⁵
80
60
40
20
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated
C.
cells/spleen x 10⁻⁶
20
15
10
5
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated

Figure 18

A


40
35
30
25
20
15
10
5
0
cells/lymph node x $10^{-6}$
noncastrated
castrated
control
2 weeks
4 weeks
time after treatment

B.

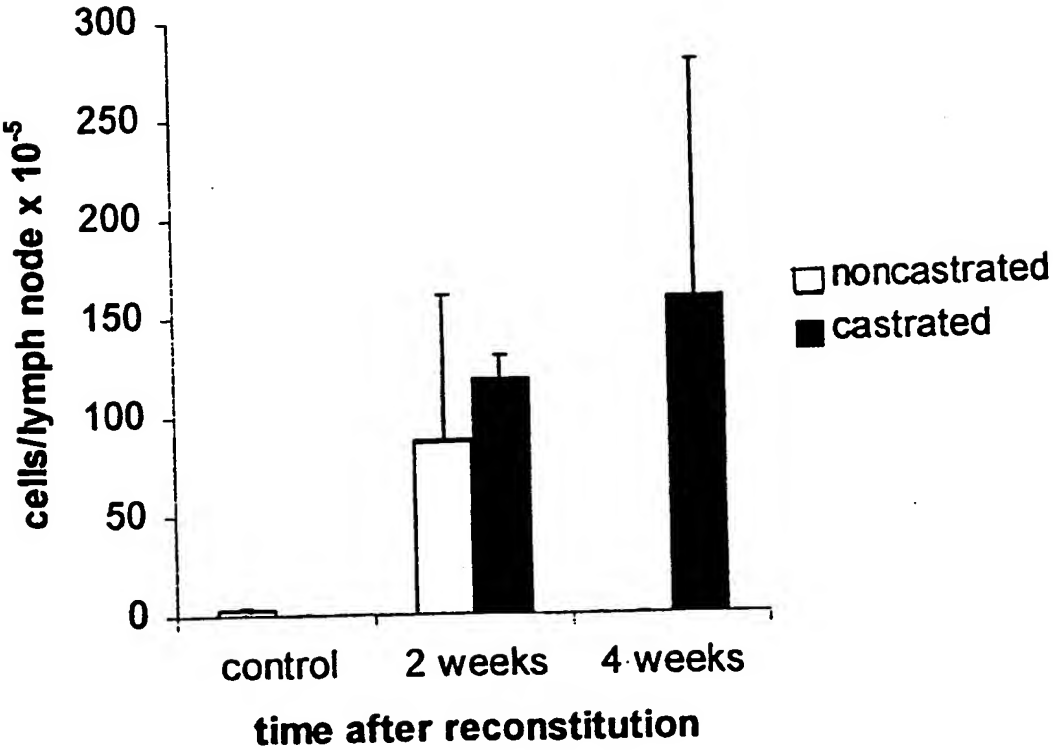

300
250
200
150
100
50
0
cells/lymph node x $10^{-5}$
noncastrated
castrated
control
2 weeks
4 weeks
time after reconstitution

**Figure 19**

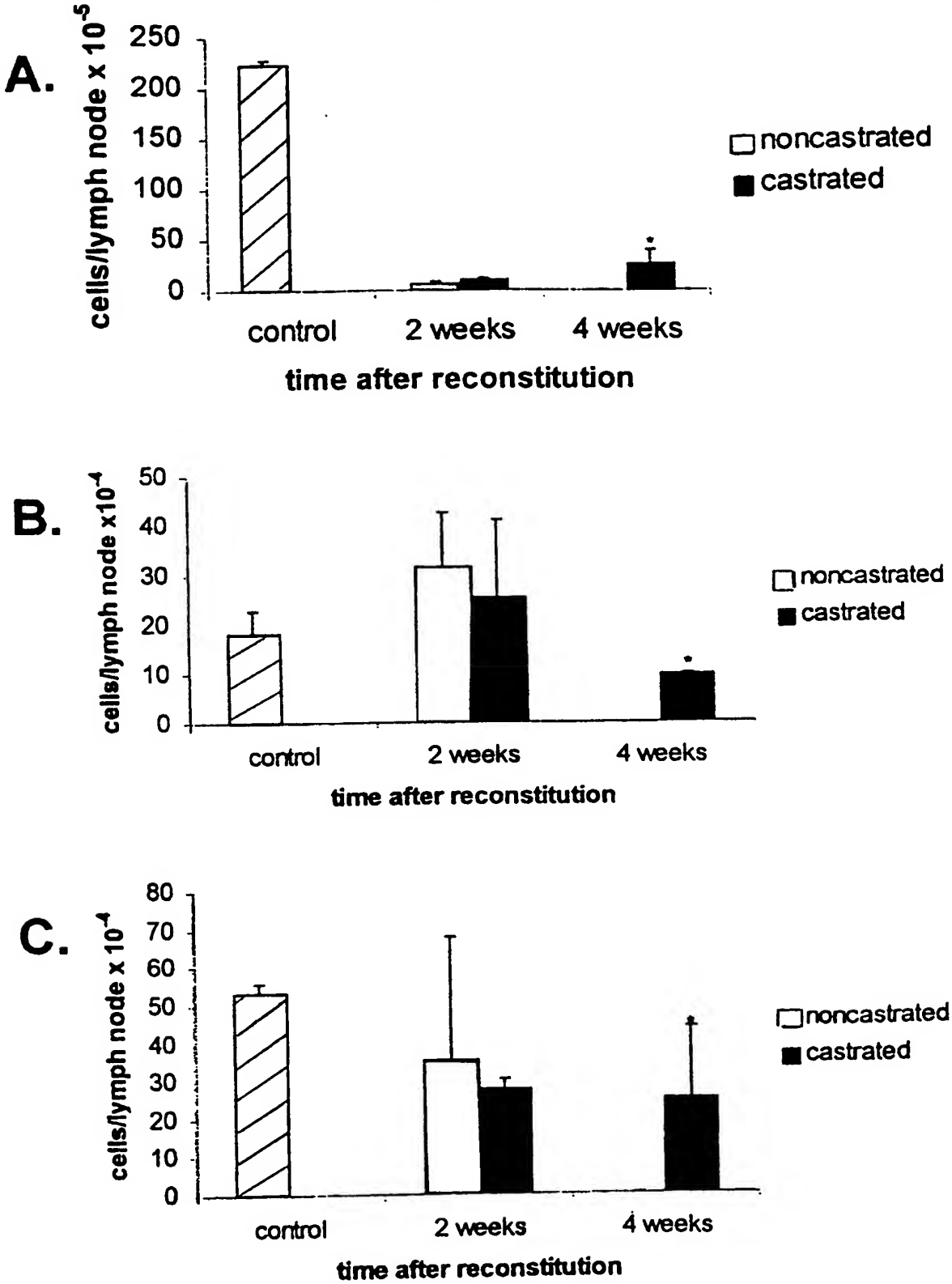

A.
cells/lymph node x 10^-5
250
200
150
100
50
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated
B.
cells/lymph node x10^-4
50
40
30
20
10
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated
C.
cells/lymph node x 10^-4
80
70
60
50
40
30
20
10
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated

Figure 20

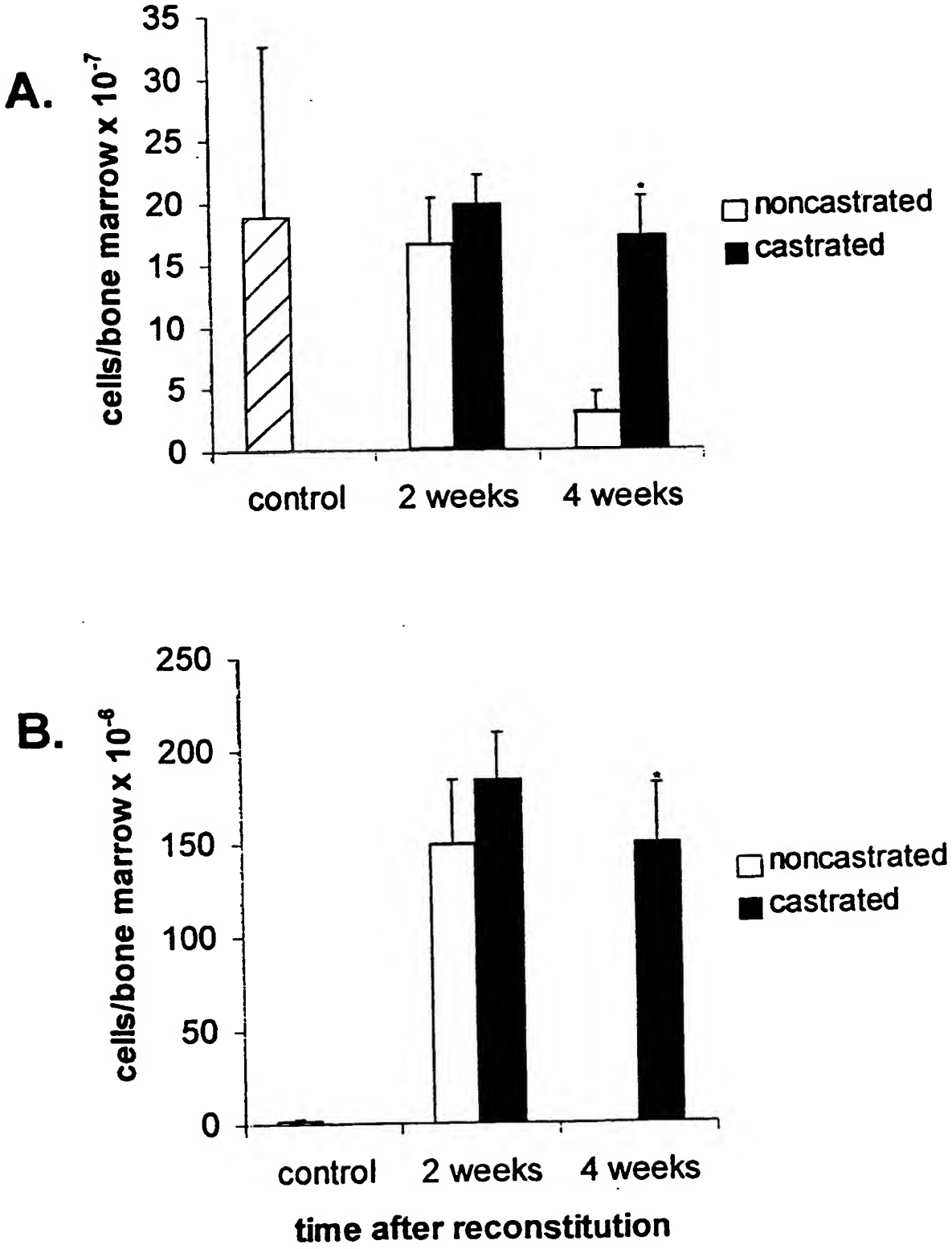

A.
cells/bone marrow x $10^{-7}$
35
30
25
20
15
10
5
0
control
2 weeks
4 weeks
noncastrated
castrated
B.
cells/bone marrow x $10^{-6}$
250
200
150
100
50
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated

Figure 21

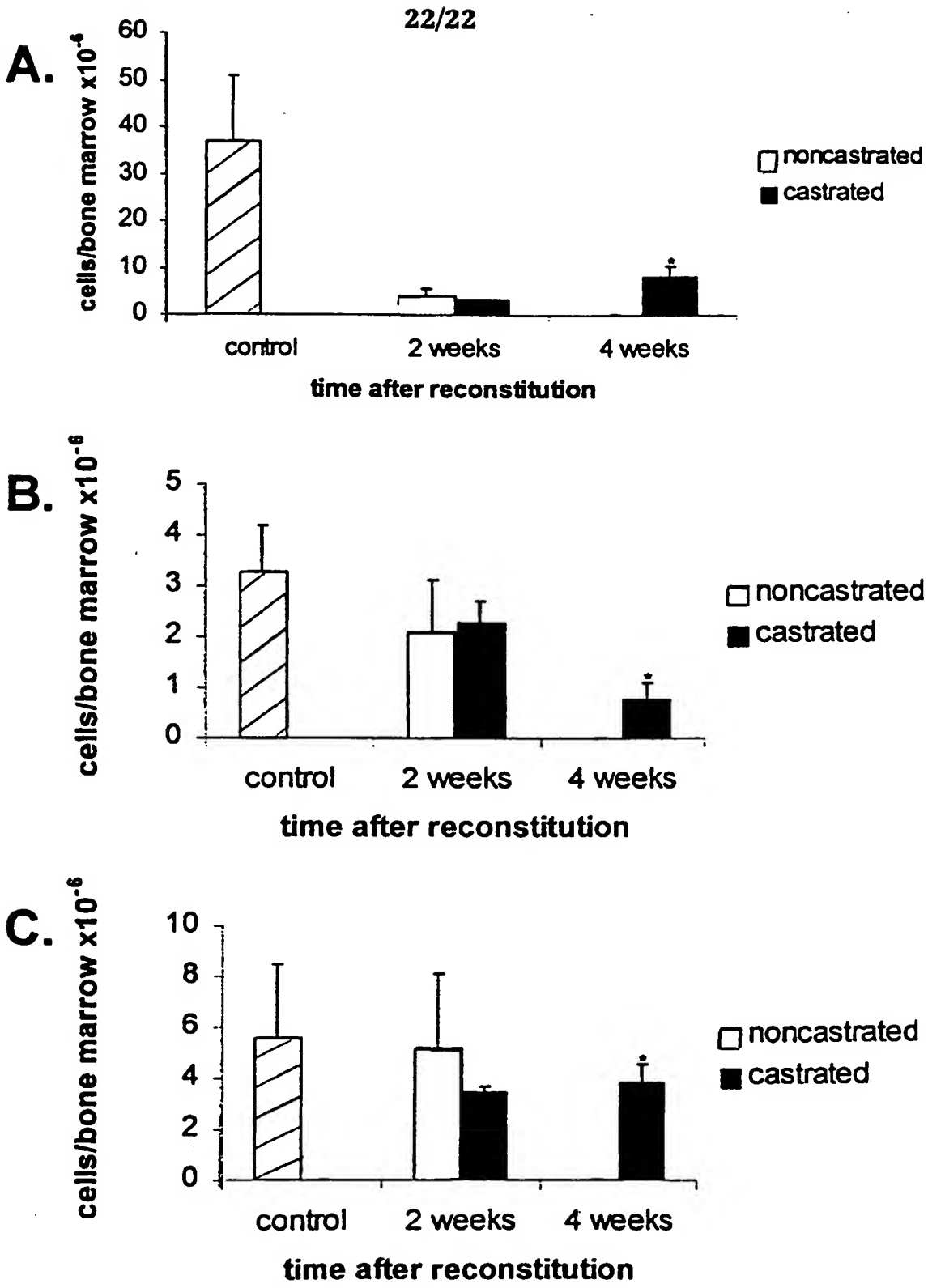

A.
cells/bone marrow x10⁻⁶
60
50
40
30
20
10
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated
B.
cells/bone marrow x10⁻⁶
5
4
3
2
1
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated
C.
cells/bone marrow x10⁻⁶
10
8
6
4
2
0
control
2 weeks
4 weeks
time after reconstitution
noncastrated
castrated

Figure 22